Implementation of morphology and genetics to more clearly elucidate classification of the genus *Psilocybe* in Australia

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Abstract

In the field of Ethnomycology, clearly describing and characterising fungi taxa is a prerequisite to understanding their potential properties so that they can be studied, developed and eventually employed by mankind. Previous studies have shown that some members of the genus *Psilocybe* may be useful but of the over 200 described species and many more undescribed species, only a handful have been investigated. It is assumed that many of the described and undescribed taxa that occur in Australia because of their morphological similarities, have molecular and chemical profiles that are the same as those from overseas. This investigation is initiated to clearly elucidate the morphological and molecular status of the 'species' of Australia and provide a comprehensive taxonomy of the species from Australia and based on their combination of those two factors. Through the provision of clear identification and characterisation of these potentially useful species, studies into the development of this group of fungi for medicinal applications will be made easier and allow future researchers to more clearly target their investigations. The current study shows that the phenotypes of *Psilocybe* and morphologically similar genera have multiple evolutionary origins. All 96 samples were been identified morphologically and 88 of them genetically classified too. The results from this study will be of immense use to others working on the taxonomy of *Psilocybe* in other countries.

Key Words

Fungi, *Psilocybe*, NGS (Next Gene Sequencing), PCR (Polymerase Chain Reaction), Marker, Geneious, GLC (Genealogical concordance)

Doctor of Philosophy Declaration

"I, Homa Hemmati, declare that the PhD thesis entitled Implementation of morphology and genetics to more clearly elucidate classification of the genus *Psilocybe* in Australia, is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Except where otherwise indicated, this thesis is my own work".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

Signature  Date: 01.03.2023

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Dedication

This effort is truly dedicated to the martyrs who lost their lives for the freedom of Iran.

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4 Introduction

Ethnobotany is known as an operative way for detection of new drug candidates (Vuoristo et al. 2011). The importance of correct identification when the plants and fungi have bioactive compounds, stands out dramatically. Misidentification of mushrooms often leads to severe mushroom poisoning and leading to death, as some toxic mushrooms imitate the morphology of edible mushrooms, which are not simply distinguishable (Kakoti et al. 2021). The discovery, development and use of products sourced originally from wild fungi are the ultimate goal of any Ethnomycologist. Apart from the general human use of consumption for food, fungi of various types have been widely but tentatively applied in medicinal settings. A medicinal plant is one whose one or more of its organs contain substances that can be used for healing or which are precursors for the synthesis of beneficial medications (Ogunkunle & Ladejobi 2006). Fungi play critical roles in carbon and nutrient cycling of terrestrial and aquatic ecosystems, and they are important pathogens and mutualists (Truong, DT et al. 2017). Most notable studies have investigated the use of fungi-derived chemical compounds in psychoanalysis and treatment to assist in the breaking of a person's habitual experience of the world. These compounds can help patients caught in an ego-centred problem cycle and assist them in release from their fixation and isolation (Schultes & Von Reis 1995). There are some related and possibly psychotropic *Psilocybe* species with unresolved systematic positions. Due to lacking both a morphological comparison and sufficient molecular data, many details about these species remain undetermined. This is an immense confusion that need to be fixed. Thus, a taxonomic re-evaluation of these important fungi is urgently needed.

Genetic data provide researchers with an incredible capability to recognise species, illuminate phenotypic plasticity, link life-cycle stages, infer phylogeny, and agree to an exploration of the geographical variation far beyond that which can be concluded from morphological data only. Molecular techniques are helpful in delineating boundaries between species when there are no obvious morphological difference (Blasco-Costa et al. 2016). Folk taxonomy (vernacular

naming systems) is distinguished from scientific taxonomy in that it remains within social relations and is therefore restricted to cultures and/or regions.

Species identification based on combined morphological and molecular genetic analysis is an important step towards understanding macromycete (fungi forming fruiting bodies) diversity, ecology and phylogeny. In many disciplines, obtaining accurate taxonomic identifications can be imperative. Morphological and anatomical data should always be compared with the inferences from molecular data sources.

The field of Ethnomycology is well established and has been the focus of many concerted studies over many decades (Trappe, Claridge, Claridge, et al. 2008). A phylogeny is an explanation of how sequences evolved, their genealogical relationships and therefore how they came to be the way they are today. A phylogenetic tree shows the inferred evolutionary relationships among various biological species based on similarities and differences in their physical or genetic characteristics. These relationships are discovered through phylogenetic inference methods such as DNA sequences. Taxonomy is the classification, identification and naming of organisms. Species delimitation is the act of identifying species-level biological diversity. As genetic data have become easier and less expensive to gather, it has experienced a variety of methodological approaches to species delimitation.

Today's threats to biodiversity from habitat loss and climate change are occurring at an unprecedented scale, and it is possible that many species may become extinct before they have been discovered (Truong, C et al. 2017). A few medically important taxa, including members of the genus *Psilocybe,* have been investigated and segregated by their chemical constituents. There remain many unanswered questions on the classification of members of the medically important genus *Psilocybe* in Australia and whether these taxa can be effectively and more clearly classified using a combination of two distinct methods (morphology and molecular).

Systematics has two major goals:

1) Discover and describe species

2) Determine the phylogenetic relationships of these species

However, there is no report for such conceptions in fungal taxonomy. Another significant concern is that the number of novel phylogenetic lineages and new Phylotypes is on the rise. A combination of microbiological and molecular methods reveals some novel phylogenetic clades that constitute new groups of fungi.

Existing methods for delimiting species using non-genetic data like chemistry, morphology and ecology stay miserably understudied. Various arrangement of data-form morphology, genetics, geography and ecology are approved as normal information for species delimitation studies. In practice any study relating different forms of data to support theories of species boundaries including mapping morphological characters into a molecular phylogeny, can be measured integrative. Integrative techniques for species delimitation fall through a wide range alternating from verbal and qualitative assessments of data modules to quantitative techniques that approve altered data sorts to contribute to statistical species delimitation. In most taxonomic studies employing both molecular and morphological data proficient estimate in evolutionary use to some point to evaluate the definitive status of nominee species. In spite of the possible trials and restrictions of using phenotypic data, these characters have delivered a plethora of appreciated evidences for accepting species boundaries (Leavitt, Moreau & Thorsten Lumbsch 2015).

Fungal species identification based on genetic analysis is a promising alternative to morphological and physicochemical studies. Molecular taxonomic methods are the most broadly acknowledged tools for reliable identification of the proper taxonomic level at which it would be most revealing and its association with morphologically definable taxonomic groupings. Fungal taxonomists use these physiological and biochemical tools for the identification and classification purpose. Therefore, it can be granted that phenotypic characters cannot always be distinct between taxa and the aid of molecular systematics will be useful in delimiting unclear species boundaries.

Nomenclature is the rules governing the naming of taxa. PhyloCode is the new nomenclature code which is the International Code of Phylogenetic Nomenclature for the formal set of rules governing naming clades. The techniques for evaluating the structures of nucleic acids (DNA and RNA) have made additional characters open to systematic mycology. Nucleic acid technologies have delivered new insights into the evolutionary interactions and the diversity among mushrooms (Borovička et al. 2011).

Genetically, fungi have marginally larger and more complicated genomes than bacteria. Like humans, they have introns, but they are only about 1/300th the size of introns in *Homo sapiens* (Thacker 2003). The fungi kingdom is among the most diverse eukaryotic lineages on earth with estimates of several million extant species. More than 80% of plant species form symbioses with fungi and these symbioses have been crucial to the colonization of terrestrial ecosystems (Truong, C et al. 2017). Fungi are the most abundant and diverse organisms on earth (Jones & Richards 2011). However, a substantial amount of the species diversity, relationships, habitats, and life strategies of these organisms remain to be discovered and characterized. One important factor hindering progress is the difficulty in correctly identifying fungi. Despite their impacts on primary ecosystem functions, assessments of fungal biodiversity estimate that only 10% of fungal species have been described (Jones & Richards 2011).

Mushrooms are widely used for various purposes. Species in the Agaricaceae are broadly recognized due to their medicinal and nutritional properties. Nutritional profile of mushrooms includes total carbohydrate content ranging from 26 to 82%, largely made of carbon, starches, pentoses, hexoses, disaccharides, amino sugars, sugar alcohols and sugar acids. This is in addition to their high fibre content, low lipid levels that is made up of polyunsaturated fatty acids and absence of cholesterol. Others uses include dyeing of wool and other natural fibres, medicine, food, and dietary (Tidke & Rai 2006).

It is essential to be able to identify accurately the fungi taxa under investigation. *Psilocybe* fungi contain diverse bioactive compounds that are of potential use to humans, especially in the fields of medical and psychiatric pharmacology. *Psilocybe* fungi have been used for spiritual and therapeutic purposes for

hundreds and probably thousands of years. More recently, with the development of advanced analytical techniques, the bioactive compounds have been isolated and identified, and used to treat disorders. These compounds are taxon-specific, so are restricted to certain genera and species. The genus Psilocybe has long been recognised as the source of psychoactive compounds with therapeutic effects, but its taxonomy is poorly known. More than 200 species have been described, but hundreds more remain undescribed.

4.1 Morphology

All previous studies on *Psilocybe* genera relied heavily on morphological characters as the main criteria for generic delimitation without considering their evolutionary relationships, so it's a phenetic classification. It is necessary to use molecular techniques to avoid misidentification of fungal isolates. Genetics have given insight into the confused state of mushroom taxonomy by providing more accurate identification (using molecular methods). Morphological analysis, a standard method for fungi species identification, does not always produce reasonable outcomes. Due to the dependability, speed and reducing cost of DNA analysis, genetic approaches are a fascinating alternative for defining the species of biological material (Zuber, Kowalczyk, Sekuła, et al. 2011). Simple band-counting technique is used for differentiation, while phylogenetic information can be retrieved from allelic frequencies and other genetic interpretation data (Rai et al. 2014). Classification of fungi based only on anamorphic features is often integrally artificial, however, and may not therefore reflect real phylogenetic relationships. For these reasons, molecular approaches should be particularly beneficial for genetic identification of fungi and for starting their taxonomic relationships (Vilgalys & Hester 1990). The ubiquity, high diversity and frequently cryptic manifestations of fungi often dictate the use of molecular tools for detecting and classifying them in the environment (Osmundson et al. 2013). Classification system for gilled fungi and their allies (Basidiomycota) is gradually trusting on molecular data (Hussein et al. 2014).

Psilocybin producing (PS+) mushrooms are well-known as hallucinogenic mushrooms. Psilocin and psilocybin are controlled substances under Schedule 1 of the 1971 UN Convention on Psychotropic Substances; as a result, all Member States treat them accordingly (https://www.unodc.org/pdf/convention_1971). On the other hand, the regulation of the mushrooms is not handled in the same way across Europe. The potentially psychoactive properties of fresh mushrooms are not treated as illegal, but treated mushrooms are illegal. The best way is 'cultivation of any plant for the purposes of manufacture a psychoactive material' is forbidden (Stamets & Weil 1996). Lastly, a number of countries remain with unclear legislation (The European Monitoring Centre for Drugs and Drug Addiction) (EMCDDA). In many countries' drug laws, there is an unlimited pact of uncertainty around the lawful status of psilocybin mushrooms, along with a strong part of careful administration in some places. Growing of psilocybin mushrooms (psilocin prodrug) is considered drug production in most authorities and is often strictly fined, however some countries and one US state have ruled that cultivating psilocybin mushrooms does not meet the requirements as manufacturing a controlled material (Stamets & Weil 1996).

Definite and clear identification of the taxa in the fungi genus *Psilocybe* based on two methods of identification will be essential to allow full development of standards and implementation of testing methods by national and international drug control legislation. Identification of particularly useful species allows for the development of lab-based populations of known high quality 'clones' that can be further developed for clinical trial and use. Depending on the outcome of any drug trial the identification of morphologically and genetically of species of *Psilocybe* from Australia could lead to new patentable drugs. Definite and clear identification of the taxa in the fungi genus *Psilocybe* based on two methods of identification will be essential to allow full development of standards and implementation of testing methods by national and international drug control legislation. Identification of particularly useful species will allow for the development of lab-based populations of known high quality 'clones' that can be further developed for clinical trial and use. Differences in the psilocin and

psilocybin contents of the fruit bodies be influenced by the factors such as species developmental phases, climatic circumstances and the accessibility of soluble nitrogen and phosphorous in the soil (Tsujikawa et al. 2003).

Four decades after discovery of fruit bodies of *Psilocybe*, indole alkaloids of the tryptamine type have become biochemically key drugs in psychotherapy and psychodiagnostics (Keller et al. 1999a). Psilocin and psilocybin both demonstrate native fluorescence they are electrochemically dynamic (Christiansen & Rasmussen 1983). Psilocin, Psilocybin, Ibotenic acid, Muscimol and Aeruginascin are the known alkaloids in the genus of *Psilocybe*

(Matsushima et al. 2009). Two different alkaloids in Hallucinogenic Mushrooms (psilocin and psilocybin) have been observed thoroughly in recent years in order to terrace their hallucinogenic effects on the human brain (Sticht & Käferstein 2000). Maximum amounts of alkaloids are found when fresh material is freeze dried. In dried mushrooms just 1-2 % alkaloids are found (Laussmann & MeierGiebing 2010). Among these alkaloids PEA (phenylethylamine) is the main component seen in the chromatograms (Beck et al. 1998). The volume of Beaocystin (the third compound) is noticeably higher in the cap than the stipe same as Norbaeocystin, the content of psilocybin on the other hand is of the similar order of scale in both parts of the mushroom. Rather large amounts of psilocybin are also set up in dried old mushrooms which presented that the mushroom can still be potent after extended storage. Variances in the psilocin and psilocybin contents in hallucinogenic mushrooms depends on aspects such as developing stage, climate circumstances and the obtainability of soluble nitrogen and phosphorous in the soil. *Psilocybe* is considered to be neurotropic due to the bluing feature of the basidiomes just the once injured or touched. The bluing colour is the oxidative reaction of key toxins involved psilocin and psilocybin (Rossato et al. 2009). Also, the existence of an unknown compound architecturally associated to amphetamine is specified in some of this type of mushrooms (Beck et al. 1998).

Consumption of small quantities of psilocin and psilocybin in the mushrooms includes ecstasy and overindulgence or ingestion with other drugs could be lethal (Ludemann et al. 2004). “Trippy” symptoms produced by consumption of

psychoactive mushrooms begin to occur within 20-40 min after ingestion or from 5 to 10 min when prepared in the formula of soup or tea. "Mind-melting" feelings persist for up to 4-8 hours after ingestion. The hallucinogenic effects are like those observed after LSD intake (Rossi et al. 2022). However, the dose of psilocybin/psilocin essential for an equal effect is roughly 200 times greater than that of LSD. The standard dose of psilocin required to induce psychedelic effects ranges from 8 to 10 mg (Musshoff, Madea & Beike 2000). Several psychoactive agents pass over the blood-brain barrier and act on neurotransmitter receptors. The trace of psilocin and psilocybin can be followed in plasma urine, but there is no physical dependence (Matsushima et al. 2009). There are considerable differences between the symptoms detected after psilocybin ingestion and mushroom intoxication it terms of consumption poisonous mushrooms. Optical hallucinations and euphoria happen less regularly after mushroom ingestion (Beck et al. 1998).

These hallucinogenic mushroom genomes will work as models in neurochemical ecology, evolving the (bio) prospecting and synthetic biology of novel neuropharmaceuticals. Secondary metabolites are small molecules that are broadly engaged in defence, competition, and signalling among organisms. Due to their physiological activities, secondary metabolites have been accepted by both ancient and modern human cultures as medical, spiritual, or recreational drugs (Dines et al. 2015). However, prohibition since the 1970s has restricted advances in psilocybin genetics, ecology, and evolution. There has been a current resurgence of research on hallucinogens in the clinical setting; brain state imaging studies of psilocin exposure have recognized changes in neural activity and interconnectivity that underlie subjective experiences, and therapeutic trials have explored psilocybin's potential for treating major depression and addictive disorders (Reynolds et al. 2018).

Psilocybin mushroom spores are legal to have in the United States. The reason is that psilocybin and psilocin (the active compounds in psychedelic mushrooms) are registered in Schedule 1, not the mushrooms themselves. Hypothetically any plant or mushroom which contains psilocybin may be illegal. Yet, mushroom spores do not contain psilocybin or psilocin. Although the production, usage and supply of psilocybin is illegal all over Australia. (*https://www.erowid.org/*). Magic

Mushrooms had a strong role in ancient societies like Mayan many years ago (McGuire 1982). Bluing species have a significantly various habitat for instance litter, muddy soil, rotten wood or dung (Guzmán, G et al. 2012). A Mother Nature and quantity of phytochemicals show a discrepancy according to environment (Yadav et al. 2010). The fallouts show that cultivated and wild edible mushrooms are rich in health-promoting phytochemical compounds (Wandati, Kenji & Onguso 2013). Psychedelic drugs are poorly explored area and known mainly as illicit drugs. It seems certain that many uses of these fungi await discovery. Despite the interest in the activity of psilocybin, our knowledge about its distribution in the genus is poor. The botanical morphological characteristic features of some common ethnomedicinally important species of *Psilocybe* highlights the fact that not enough is known about this genus.

In relations of recorded history, medicinal plants have been in use for the past fifty decades which until the last two and half centuries was the key source of treatment to man and his domestic animals. Herbal remedy which is the use of therapeutic plants or drugs from medicinal plants in the treatment and therapy of sicknesses and diseased condition, has been with man ever since the beginning of time (Ogunkunle & Ladejobi 2006) like Ayurvedic plant which is used in numerous traditional medicines to cure a number of illnesses (Yadav et al. 2010). It is notable that the recreational and medicinal are two neighbouring areas. Humans have used psychoactive mushrooms for remedial, recreational, religious and ritual drives ever since pre-history. The potential effect of these hallucinogenic alkaloids on human brain in terms of medicinal consequence would be probable concern for new world (Matsushima et al. 2009). Intractable nervous diseases, management of alcoholism, antisocial disorder depression, schizophrenia, autism and obsessive compulsive disorder (OCD) are some of the illnesses can be treated by these mushrooms as they boost the brain's connectivity. The clinical signs of OCD are obsessive thinking, severe anxiety and distress subsequent from frequent and nonstop thinking and compulsive deeds repetitive actions supported to alternative anxiety and distress (Matsushima et al. 2009). The neurophysiological effects have been linked to the improvement of catecholaminergic activity (Beck et al. 1998). Psilocin and psilocybin are tremendously promising for the treatment of nervous disease

which involves long term treatment (Matsushima et al. 2009). The serotonergic scheme is through to mediate the neurophysiological reactions to hallucinogens (Beck et al. 1998).

The imbalance of excess free radicals in the structure is recognized as oxidative stress. It interferes with cell reliability hence typical functioning is altered leading to many stress-related diseases like cancer and diabetes. Antioxidants, such as phenolic compounds, alkaloids, saponins, flavonoids, tannins, sterols, triterpenes, coumarins and cyanogenic glycosides, seem to wipe the free radicals created in the ordinary natural absorption of aerobic cells generally in the form of Reactive Oxygen Species (ROS) (Wandati, Kenji & Onguso 2013). Although studies on a range of non-mental health issues including some cancers where treatment using fungal-based compounds have been carried out, these studies are less familiar (Penicillin excepted) but have had notable clinical and real-life impacts (Grob & de Rios 2013). There are different medicinal applications for members of the genus *Psilocybe* around the world based on their chemical components (Moreno et al. 2006). Psychoactive agents like psilocin, psilocybin, ibotenic acid and moscimol pass through the blood –brain barrier and act on neurotransmitter receptors (Matsushima et al. 2009). Even though the possible use of the compounds found in the genus *Psilocybe* have the potential for more widespread use and application, the development of these useful compounds is limited by the reliance on wild-source materials that lack quality control and full characterisation of their chemical profiles and concentrations. Initial work first started in the 1950s showed clear clinical applications of the chemical compounds found in *Psilocybe* but little work has been done to determine and clarify potentially useful or useless taxa and potentially dangerous ones (Grob & de Rios 2013) particularly in Australia.

Traditionally, specimen-based taxonomic studies have been the only way to discover new species. Because most fungi have microscopic life-stages and convergent morphological features (Lumbsch et al. 2011, Rivas Plata, Lücking & Lumbsch 2012), many fungal groups remain severely under sampled. DNAbarcoding and high throughput sequencing methods have provided a new framework for studying fungal biodiversity (Fierer et al. 2012, Schoch et al. 2012, Myrold, Zeglin & Jansson 2014) and diversity estimates based on environmental

sequences have increased exponentially. Although these ‘sequence-based classification and identification’ methods are a powerful means to rapidly detect hidden diversity, careful interpretation of these data is needed to make accurate inferences (Kõljalg et al. 2013, Lindahl et al. 2013, Hibbett, D et al. 2016). In particular, many environmental sequences cannot be associated with a known fungal species or lineage. This remains a major challenge to decipher fungal community composition and understand ecological roles of fungi in leaf litter, soil, or inside plants (Yahr, Schoch & Dentinger 2016). In some cases, these fungi are truly undescribed and their ecological roles are unknown but in other cases they represent described taxa for which no sequence is available (Nagy et al. 2011, Nilsson et al. 2016). In some circumstances, however, morphological principles present some problems and fail to resolve taxonomic relationships. This is right in cases where morphological characters are insufficient, convergent, reduced, lost or overlapping. As a result, many taxonomists have combined accessible morphological characters with biochemical or molecular characters to clarify taxonomic relationships, as well as to conclude phylogenies among fungal species.

Species are difficult to identify from morphological characteristics alone. Applying molecular techniques allows us to distinguish between species and to reveal cryptic species. Numerous molecular techniques that have been applied effectively in fungal systematics and the application of DNA sequencing joined with phylogenetic analysis, have significantly extended, due to the ever-increasing total sequence data obtainable from many organisms. Molecular characters offer substantial possibilities, as they do not simply close the gap between the traditional and molecular techniques, but also define relations between uncultured and cultured fungi (Jeewon & Hyde 2007). Several types of molecular techniques have been used to aid in delimiting species. Accurate species delimitations are of great importance for effectively characterizing biological diversity. Our criteria for delimiting species have changed dramatically over the last decades with the increasing availability of molecular data and improvement of analytical methods to evaluate these data. Whereas reciprocal monophyly is often seen as an indicator for the presence of distinct lineages, recently diverged species often fail to form monophyletic groups. At the same

time, cryptic species have repeatedly been detected in numerous organismal groups. The increasing availability of DNA sequence data enables exciting new opportunities for fungal ecology. However, it amplifies the challenge of how to objectively classify the diversity of fungal sequences into meaningful units, often in the absence of morphological characters. The development of coalescent based and other multilocus methods for species delimitation has facilitated the identification of cryptic species complexes across the tree of life. Identification of the species is faced by molecular techniques and based on morphological characters. Morphological and molecular characteristics have been applied in such tasks. Later, DNA barcoding has emerged as a new method for the rapid and reliable identification of species. Species delimitation is the method of classifying how individuals and populations fit into natural species-level clusters and not basically builds of classification. Due to the fact that species delimitation studies every so often integrate a considerable biogeographical or ecological constituent, ecological role plays an increasing part in phylogenetic and taxonomic investigation. Related functioning species delimitation approaches, the majority of which are dependent on molecular phylogenetic data united with population genetic study (Leavitt, Moreau & Thorsten Lumbsch 2015). Although identification by the traditional morphological criteria is very effective at the generic level, the large number of existing species has created a need for alternative approaches. Molecular methods have been used to improve the understanding of the natural taxonomy in fungi. Most currently available species delimitation methods are unable to accommodate non-genetic data source in a statistical framework. This is why multimarker species delimitation is an outstanding method these days to identify the boundaries of fungal taxa. Molecular biologists are nowadays playing a substantial if not principal role in species discovery. Genealogical concordance tactic uses multiple unlinked loci to evaluate the boundaries of reticulation (Hibbett, DS et al. 2011). We concur that the Genealogical concordance (GLC) delivers an applied resolution to the species delimitation (Leavitt, Moreau & Thorsten Lumbsch 2015). Phylogenetic approach to recognize fungal species is based on concordance of multiple gene genealogies the strength of Genealogical concordance phylogenetic species recognition (GCPSR) lies in its association of more than one gene genealogy. Gene genealogy concordance is well appropriate to categorizing hybrid

individuals since hybrids should group with different species in different single gene genealogies and lie at the base of the tree in pooled gene genealogies (Taylor et al. 2000).

In a numeral of studies of lichen–forming fungi a genealogical concordance benchmark has been used to mutually delimit formerly unrecognized species level ancestries and certify some conventional phenotype-based species (Leavitt, Moreau & Thorsten Lumbsch 2015). These studies reveal that phylogeny by genealogical concordance is well-matched to fungi and probable to become very widespread with mycologists. The integration of different methods and techniques should lead to the identification of usual markers for the standardization of global taxonomical studies of fungi species. Phylogenetic analysis of sole gene can be used to cluster individuals into monophyletic groups but not to rank them into species. Phylogeny can avoid the bias of defining the limits of a species by trusting on the concordance of more than one gene genealogy and removing the ambiguity about the restrictions of species in a solitary gene genealogy (Taylor et al. 2000).

Recent advances involving DNA molecular biology combined with morphology have led to the development of mixed methods, employing both morphological and molecular way to investigation. The phylogeny based on DNA sequence data and augmented by morphological data provides an informal classification manner. Raw plant materials should be authenticated by DNA bar-coding or Genetic assays before their use for the preparation of ethnomedicines that very few have used actively-guided fractionation to isolate the compounds associated with a specific ethnobotanical use. Any sort of fungal identification DNA sequenced-based identification is a reiterative process that may require further investigation and consideration of other factors including morphology and ecology. Exotic and wild mushrooms enclose phytochemical combinations are compulsory for a healthy body (Wandati, Kenji & Onguso 2013). These metabolite for use as therapeutic agents needs much attention as it necessitates careful identification and selection of species before the screening of metabolites for desired industrial applications.

To date many molecular techniques have been developed for studying population genetic diversity. When two species are related and morphologically very similar this hampers their correct taxonomic identification. The genetic situation however offers the possibility to use gene sequencing as a diagnostic tool to differentiate. Molecular methods may be used to differentiate between fungal strains with similar morphology. Next gene sequencing is used for phylogenetic purposes as well as Whole genome sequencing (WGS) and massively paralleled sequencing (MPS); one of several high-throughput approaches to DNA sequencing. As these approaches detect differences at different phylogenetic levels the obtained results can supplement each other.

Throughout the former few years traditional schemes of assessing genetic variability in the middle of strictly correlated species have been progressively supplemented by molecular approaches (Yadav et al. 2010). Shared molecular phylogenetic and morphological lines have been shown to be priceless in the diagnosis (Cheng et al. 2008). Generating sequences is nowadays becoming easier (cost and time) than analysing and publishing them (Faure & Joly 2015). Native Bayesian classifier tool presented by Ribosomal Database Project (RDP classifier) uses a very fast algorithm, recognized on the Bayes' theorem proper for the analysis of large volume of sequence data (Bacci et al. 2015). NGS methodologies with the ability to generate millions, even billions of sequence reads over the course of a few hours to days, are the common molecular technique in phylogeny (Nilsson, Abarenkov & Kõljalg 2016).

Classification of fungi based on a specific set of morphological characters leads to disagreement and continuous readjustments in the taxonomy of many fungal groups. A fundamental incongruency exists between the molecular data and morphological studies concern the systematic classification. Some interesting similarities and differences in gene structure are revealed by a comparison of the Signature compounds with a rich diversity of ethno medicinal uses. Systematic studies using molecular characters based on molecular observations could explain some of the traditional uses of this species. The fact that this important group of fungi can be distinguished by amplifying some informative regions. To outline monophyletic genera only morphological characters, need to be combined

with all that is famous about a group of species as well as their biology morphology and the outcomes of molecular studies (Rossman 1996). Molecular studies are increasingly contributing data that are useful to species classification. Detailed molecular studies are needed with the integration of the results with morphological data. So far there are no proper barriers to the publication of new taxa founded merely on morphology (Hibbett, DS et al. 2011). Integration of telemorphic taxa may be possible by combining results of morphological and molecular data.

5 Literature Review

5.1 Distribution

The 19th Century was the era of the acceptance of evolution. Darwin believed that species all trace back to the same common ancestors. However, Lamarck was of the belief of different kind of organisms traced to separate origins (Baum & Smith 2013). In fact, taxonomy should reflect evolutionary relationships. Classifying the numerous species descriptions through the world alongside with discovery of exact novel biodiversity will demand increased reliance on molecular barcoding for fitting them into the already accessible system of classification. Using molecular methods is a tool to sort out current classifications based on morphological characters. DNA barcoding includes the sequencing of a certain locus that can be used to classify fungi at the species level based on the DNA sequence. A few recent studies have also used the combination of morphological features and genetic information for identification of mushrooms properly (Kakoti et al. 2021). Classification of fungi based on a specific set of morphological characters leads to disagreement and continuous readjustments in the taxonomy of many fungal groups. Very few researchers have incorporated protein-coding loci to address lower-level systematic studies among mushrooms and their allies (Abadio et al. 2012). In nature, there are >200 fungal species with hallucinogenic / narcotic properties categorized in the genera *Psilocybe*, *Gymnopilus*, *Panaeolus*, *Agrocybe*, *Conocybe*, *Copelandia*, *Galerina*, *Gerronema*, *Hypholoma*,

Inocybe, *Mycena*, and *Plutes* that may contain the active principles ibotenic acid, psilocybin, psilocin, and baeocystin. Legal regulations of psilocybin mushrooms by country are variable, for instance it is prohibited in United Kingdom, United State, Japan, Mexico, Australia, France, Germany, and Denmark. It is legal in Brazil, Jamaica, and Canada (Solano et al. 2019).

Ethnopharmacology predominantly cast-off in 1967 in the title of a book on hallucinogens (Dews 1968). All the way through human history, societies used numerous resources from nature to cure their illnesses and improve their wellbeing. Substances are derived from flora, fauna and mineral bases originate in people's direct surrounds but also in accessible districts. Natural surroundings have been the foundation of therapeutic agents for thousands of years, and an outstanding number of modern drugs have been isolated from natural bases, many founded on their use in old-style medicine. These plant based traditional medical structure continue to perform a key role in health care, with about 80% of the world's residents depend principally on old-fashioned medicines for their primary health care, still pharmaceutical plants form the standard issue of traditional medicine. There is limitless possibility for innovative drug discovery grounded on traditional plant uses. Plenty of substantial latest drugs like digitoxin, reserpine, tubocurarine, ephedrine, ergometrine, atropine, vinblastine and aspirin have been revealed by subsequent signs from the folk consumers (Ghorbani, Naghibi & Mosaddegh 2006). Essentially all the ethnobotanical use of *Psilocybe* rests on the fact that, it protects the compounds psilocybin and psilocin both of which are hallucinogenic (Badham 1984).

Many fungal species naturally grow on dung or in pastures in subtropical to tropical climates, although the target genus *Psilocybe* does occur widely in more temperate areas (Keller et al. 1999a). In the wild, mushrooms generally emerge after a few rainy days during cool seasons (Smith & Weber 1980). 277 of 300 *Psilocybe* species rising on stems, leaves, seeds, earth, dung, sawdust, straw, deadwood and mosses (Ramírez-Cruz et al. 2013).

Initial investigations of the genus *Psilocybe* started in the 1950's and showed clear clinical applications of the chemical compounds found in *Psilocybe* but little

work has been done to determine and clarify potentially useful or useless taxa and hypothetically dangerous ones (Grob & de Rios 2013) particularly in Australia. Definite and clear identification of the taxa in the fungi genus *Psilocybe* based on two methods of identification will be essential to allow full development of standards and implementation of testing methods as recommended by national and international drug control legislation.

Identification of particularly useful species will allow for the development of lab based populations of known high quality 'clones' that can be further developed for clinical trial and use. The identification of *Psilocybe* taxa from Australia, though combined morphological and genetic methods could, after testing, lead to new patentable drugs. The discovery, development and use of products sourced originally from wild fungi are the ultimate goal of any Ethnomycologist!

Mushrooms in the genus *Psilocybe* have long been known in traditional cultures for their medicinal, neurotropic and psychoactive properties. These mushrooms are used and held sacred by indigenous peoples in Mexico and some other countries but remained relatively unknown to western science until the 20th century. Since western scientists became more familiar with this group of fungi in the 1950s, these 'traditional medicines' have been recognised and acknowledged as having potential wide-ranging medical applications in the western tradition. In traditional cultures in the native range of many of the *Psilocybe* with known chemical properties, 'Menfolk' have almost solely used *Psilocybe* mushrooms for remedial, religious and spiritual observances. The Mayans are well known to use *Psilocybe* for holy rites and in other cultures they were, and in some cases still are, called divine mushrooms (Ramírez-Cruz et al. 2013).

Worldwide, *Psilocybe* and other mushrooms with psychoactive properties are typically classified as poisonous (Matsushima et al. 2009) as they contain indole alkaloids; psilocybin (4-phosphoryloxy-N, N-di methyltryptamine), psilocin (4-hydroxy N, N dimethyltryptamine) and baeocystin (4-phosphoryloxy-N-methyltryptamine). However, in recent years these same chemicals that are classified as 'poisonous' have had their innate chemicals, the indole alkaloids, recognized as biochemically vital drugs in psychiatric therapy and psychodiagnostics (Christiansen & Rasmussen 1982). Additional chemicals,

especially the phenolic compounds, alkaloids, saponins, flavonoids, tannins, sterols, triterpenes, coumarins and cyanogenic glycosides have been found through analysis of wild source mushrooms. These compounds act to neutralise the free radicals generated in the typical metabolism of aerobic cells, normally in the form of reactive oxygen species (ROS) (Wandati, Kenji & Onguso 2013).

The main chemical found in potentially medically useful mushrooms is psilocybin. This naturally occurring compound is found in a wide variety of mushrooms, including the members of the genera *Psilocybe*, *Conocybe*,

Gymnopilus, *Panaeolus*, and *Stropharia*. Members of the fungal genera *Psilocybe* and *Panaeolus* are well-known to produce the controlled compounds psilocin and psilocybin (Linacre, A, Cole & Lee 2002). The action of psilocybin might be associated with its structural match to serotonin (Badham 1984).

Psilocybe mushrooms may produce psilocybin, psilocin and their by-products although the concentrations produced range from non-existent to high (Rossato et al. 2009). Most of the supposed psychoactive species are counted in the presently classified genus *Psilocybe* as described by (Guzmán, G 2019) (Rossato et al. 2009). Although the chemical based on the genus name found in some members of the genus, many of these species have not been thoroughly investigated even though they are considered to be neurotropic owing to the 'bluing' of the basidiomes when bruised or damaged. Typically, the bluing colour is a manifestation of the oxidative response of the key toxins elaborated — psilocybin and psilocin. Natural indole hallucinogenic (psychodysleptic) alkaloids are identified as specific Central Nervous System (CNS) (the Centre for Neurobehavioral Genetics) serotonin receptor inhibitors affecting amplified neuronal serotonin with synchronized inhibition of serotonin release from presynaptic stations (Borowiak, Ciechanowski & Waloszczyk 1998).

Uses of the chemical psilocybin have been documented and range from minor, whole *Psilocybe* being used for weak fever, cold and toothache (Schultes, Hofmann & Rätsch 2001) to more profound where the chemical is one of a small group of chemicals capable of passing through the blood-brain barrier and having intense effect on the principal nervous system (neuro-transmitter receptors). Interestingly, psilocybin and psilocin have shown therapeutic success for

obsessive –compulsive disorder (OCD), which is a notoriously hard to-treat nervous illness (Matsushima et al. 2009). The study of molecular systematics of the larger group of psilocybin mushrooms supports separation of taxa from within the present genus *Psilocybe* (Rossato et al. 2009). *Psilocybe* belongs to the much larger Agaric family Strophariaceae which contain both highly chemically active species such as Death Caps (*Amanita phalloides*) as well as the most economic food species, the Common Edible Mushroom (*Agaricus bisporus*) (Boekhout et al. 2002). *Psilocybe* is a widely distributed genus and consists of approximately 150 potentially chemically active species, amongst the 227 found worldwide.

Some gene sequences are extremely conserved since they develop gradually then can be used in systematic revisions for indistinctly entity as well as in *Psilocybe* (Lee, Cole & Linacre 2000a). A molecular built line described by total DNA extraction, amplification and sequence analysis of an isolated region of the rDNA gene can be useful to identify *Psilocybe* species (Gambaro et al. 2015). The members of the same species create the same band outline, the members of the same genera in different species create different general band and different genera create completely different band design. Random Amplification of Polymorphic DNA (RAPD) is the first method of molecular classification which scans the whole genome in one response (Linacre & Graham 2002).

Psilocybe (Basidiomycota / Agaricales / Strophariaceae) (Borovička et al. 2011) belongs to the agaric family Strophariaceae (Boekhout et al. 2002). *Psilocybe* as currently understood is a polyphyletic set composed of two separate genera.

The name *Psilocybe* is nowadays used for the clade of psychoactive species (Ramírez-Cruz et al. 2013). Species of *Psilocybe* s.str. contain psilocybin, psilocin and baeocystin yet *Deconica* has none of these compounds despite the fact that the name *Deconica* is applied to those ex species of *Psilocybe* that lack hallucinogenic compounds (Ramírez-Cruz et al. 2013). *Deconica* (Basidiomycota, Agaricales, and Strophariaceae) is a genus of agaricoid fungi and includes nearly 42 species (Index Fungorum; http://www.indexfungorum.org). Many *Deconica* species were often considered members of the genus *Psilocybe* but *Deconica* has been newly separated from *Psilocybe* by phylogenetic analyses using

nuclear large ribosomal subunit rRNA, 5.8S rRNA, and RPB1 markers. Although *Deconica* shares several morphological characteristics with *Psilocybe*, it can be distinguished from *Psilocybe* by multigene-based phylogenies (Park et al. 2017). It is not only difficult to use morphology to distinguish between *Psilocybe* species but it can be a problem at higher taxonomic levels.

Macro- and micromorphology are very similar in *Deconica* and *Psilocybe*. There are morphological details that can support to separate the two genera. In *Deconica* the pileus is usually convex to flattened, with mostly attached lamellae with a decurrent tooth in most species, and the lamellae colour is pale to dark brown to reddish brown, hardly purple brown, but never blackish purple as in *Psilocybe* species. In *Psilocybe* the pileus is hemispherical or conical at least when young, often with ascending darker lamellae (Diagram 5.1.). The key difference between the two genera is the existence of hallucinogenic compounds found only in *Psilocybe* and it is central to the importance of correct identification (Ramírez-Cruz et al. 2020). The family placement of *Psilocybe* and *Deconic*a has been the focus of much debate. Guzman and Singer (2019) considered *Psilocybe sp.*in the Strophoriaceae. Singer acknowledged two subfamilies in Strophariaceae, Stropharioideae and Pholiotoideae, based on the basidiospore morphology and spore print colour. In Stropharioideae the basidiospores are yellowish brown and the spore print is dark brown or purplish brown. This subfamily covers the genera *Hypholoma*, *Psilocybes* and *Stropharia*. In the Pholiotoideae the basidiospores are yellowish orange or yellowish brown and the spore print has reddish, rusty-brown or red-brown tones (Ramírez-Cruz et al. 2013).

Diagram 5.1 Simple schematic of a fungus
(Consists of cap, gills, stalk and hyphae) (Ramirez-Cruz et al. 2013)

5.2 Chemical Contents

The existing advances in molecular systematics have allowed professionals to gain and analysis big plant-derived DNA data sets which once collected, resulted in a new classification of the species. This phylogenetic classification carries a leading framework to evaluate theories about the taxonomic factors (Simmonds 2000). Recent announcement of multilocus phylogenetic tactics permit the independent identification of species boundaries in the fungi (Cheng et al. 2008). The basics of the molecular identification are nucleotide sequence deviation (Nugent & Saville 2004). The molecular method can classify a comprehensive variety of fungi species (Kowalczyk, JE et al. 2015). Secondary markers must be used to perfectly account genetic diversity (Schoch et al.

2012).

Psilocybin is similar to morphine-6-glucuronide to a high degree because of the existence of a phenolic and tertiary amino group compounds. Additionally, psilocin should have similar extraction properties as morphine as the incidence of that phenolic and a tertiary amino group (Sticht & Käferstein 2000). Also, psilocybin and psilocin are comparable in structure to serotonin (the neurotransmission substances in the brain) (tryptamine), an intracerebral neurotransmitter and act on serotonin receptors in the brain (Matsushima et al. 2009). The use of psilocybin containing mushrooms was in many instances

dismissed as a frivolous and indulgent pursuit of 'counterculture types' until Albert Hofmann (the famous Swiss natural products chemist) succeeded in isolating the active tryptamine alkaloid, psilocybin, from these mushrooms (Grob & de Rios 2013). Physiologically, psilocybin adjusts brain concentrations of indole compounds as well as serotonin and so modifies brain chemistry. Brain concentrations can be changed on indole compounds including serotonin (Sticht & Käferstein 2000). Psychoactive mushrooms are usually classified as toxic mushrooms (Matsushima et al. 2009) but Psilocybin mushrooms are surprisingly non-toxic (Badham 1984).

In recent studies of the *Psilocybe* in Europe, it is demonstrated that, even stable macro– and microcharacters observed in various collections over the years, which would normally support a rather narrow species concept of *Psilocybe,* are not supported by molecular data: neither by ITS rDNA, nor LSU and EF. Taxonomic ambiguities between these taxa (*Psilocybe*) have been originated from traditional classification which is unclear due to similar morphological and ecological features. Therefore, not only morphological characters but also molecular data are needed to identify this macro-fungus (*Psilocybe*) correctly. To date, morphological analysis has been the keystone to phylogeny systematics. However, since the late-1980s we have seen an increased combination of genetic data to overcome difficulties faced when morphological data are considered in isolation (Blasco-Costa et al. 2016). The weakness of morphology is that species identified by morphology frequently include more than one species when detected by phylogeny or biology (Taylor et al. 2000). The lack of distinctive morphological characteristics means that morphology itself does not provide an adequate basis for recognition of a new taxon. Some of these distinctions have been confirmed using molecular data, it means that molecular data may be useful in determining if clear distinctions can be made. About 300 000 public fungal ITS sequences constitute a poor candidate for the base of taxonomic annotation of lately produced sequences, specifically once used in combination with completely automated pipelines. Simply around half of these sequences are annotated to the level of species. This half represents roughly 20, 000 diverse species (Kõljalg et al. 2013). As the amount of nucleic acid extracted does not necessarily reveal all the species / populations within one sample,

interpretation of bands can be problematic. Frequently, dominant bands might mask more than one species, resulting in an underestimation of diversity (Jeewon & Hyde 2007). ITS sequences available in public databases turned out to be only partially reliable. It relates to the problems of identification of these fungi. Some of the sequences used as a reference come from misidentified fungi. In addition, a number of studies involving the use of DNA fingerprinting systems do not address the evolutionary history and affinities of fungal taxa based on phylogenetic analyses. Some molecular sequences already available in databases are misidentified. This underlines just how very untidy *Psilocybe* taxonomy is! It is vital to have voucher and reference material, so you can check identifications the ITS region cannot distinguish between all species. Differentiating between species with the ITS region similarly, has other limits such as dependency on read length and incompleteness in public databanks. Therefore, it must be expected that, some environmentally derived ITS sequences should be ordered as unclear at the rank of species

(Dannemiller et al. 2014). Fully annotated ITS sequences assist universal scale Meta studies on phylogeny evolutionary and biogeography (Kõljalg et al. 2013) (Table 5.1.

Table 5.1 Australian known and unknown *Psilocybe* sp. derived from Australian Virtual herbarium

Australia known Species	Melbourne known Species	Total Number of Australia Specimens	Total Number of Melbourne Specimens	Number of Australia Unknown Specimens	Number of Melbourne Unknown Specimens
25	11	223	103	76	38
Psilocybe alutacea					
Psilocybe argentina					
Psilocybe aztecorum					
Psilocybe brunneoalbescer	✓				
Psilocybe coprophila	✓				
Psilocybe crobula	✓				
Psilocybe cubensis					
Psilocybe cyanescens	✓				
Psilocybe fimetaria					
Psilocybe formosa					
Psilocybe inquilina	✓				
Psilocybe kolya					
Psilocybe makarorae					
Psilocybe merdaria	✓				
Psilocybe mexicana					
Psilocybe montana	✓				
Psilocybe musci	✓				
Psilocybe noraezelandiae					
Psilocybe physaloides					
Psilocybe pseudobullacea					
Psilocybe sabulosa					
Psilocybe semilanceata	✓				
Psilocybe stercicola	✓				
Psilocybe subaeruginosa	✓				
Psilocybe thrausta					

5.3 Therapeutic Effects

In recent years, we have called attention to the absence of information on the relative importance of a medicinal plant (or other beneficial plants) within a culture and the necessity for comparing the use of plants inter-culturally. Such studies have important inferences for investigation in the field of natural products. In the meantime, these ethnobotanical studies point out to the species which mostly studied phytochemically and which we consider them to be the most probable ones to contain bioactive compounds. Out of 250,000 advanced plant species which are supposed to be present in the world, only limited ones have been systematically studied for their useful potential. Another important point is the loss of native data which has an influence on modern medicine (Ghorbani, Naghibi & Mosaddegh 2006).

There may be species in the above genera that are presently not catalogued or described which occur in Australia and that have properties which are superior to those species already described and characterised. It is well known that there are potentially many new 'species' of *Psilocybe* in Australia and hypothetically several segregate genera. There has been a resurgence of study on these types of mushrooms in various countries, most notably, Japan, Mexico, Brazil, Nepal and Thailand (Matsushima et al. 2009; Rossato et al. 2009). John Hopkins Institute has been working on dosage levels of isolated and purified psilocybin to achieve maximum benefit in reducing fear and anxiety in cancer patients (Johns Hopkins University Psilocybin and Cancer)

(www.heffter.org/research-jhuc.htm). Most of these studies rely on chemical analysis of the most commonly known species of the genus, *Psilocybe cubensis*. It is not known, what the phylogenetic profiles (phylogenetic profiles describe the presence or absence of a protein in a set of reference genomes) are for the vast majority of the 227 known species or the presently countless numbers of undescribed or recognised species. On the other hand, still not a single article on the topic of ethnomycological knowledge transfer can be found in the systematic literature apart from one conference abstract (YaminPasternak 2018).

Mushrooms are similarly examples of nutraceuticals (food with medicinal value), both according to traditional remedial systems and in the light of the new discoveries regarding the immunostimulatory part of fungal polysaccharides

(Łuczaj & Nieroda 2011). In general, the manufacture of secondary metabolites that are supposedly valuable for pharmaceutical and agricultural applications is well-known among fungi (Cheng et al. 2008). The hallucinogenic activities of psilocin and psilocybin are 1/200 of LSD and 50 times greater than that of mescaline (Maruyama et al. 2003). The chemical similarity to serotonin, one of the neurotransmission elements in the brain, defines their hallucinogenic effects (Maruyama et al. 2003). Two polar sets of psilocin, a phenolic and a NH-group by trimethylsilyl (TMS) could avoid thermal disintegration. Bluing occurrence in most fresh psilocybin mushrooms when cut or injured, oxidize and go blue to purple within 30 to 60 minutes (Sticht & Käferstein 2000). The greater the psilocybin content in a distinct sample, the slighter the psilocin content (Laussmann & Meier-Giebing 2010).

This study will allow for a direct comparison of previously undescribed species with species putatively allocated to the genus *Psilocybe* that are already profiled and will identify potential candidates for further investigation and study for their potential as a source of bioactive compounds.

6 Research question

In the need to describe and protect as many species as possible we addressed the following main questions:

What are the best methods to rapidly document fungal biodiversity? Are traditional, specimen-based approaches still useful?

Based on a combination of morphological and genetic profiles, what are the relationships and classification of the taxa within Australian '*Psilocybe*' and how do these taxa relate to modern understandings of the classification and phylogeny of the kingdom Fungi?

The question will decide the objectives on which the methodology will be based.

Another significant question is: which genes and what features of that genetic sequence are critical, useful and reliable to classify uncultured fungi? While rDNA offers sufficient variability for evolutionary and phylogenetic inferences, should more genes be sampled?

7 Research Hypothesis

We can identify *Psilocybe* taxa by their morphology and genetics profiles. We hypothesize that the groups with near identical sequences actually are single species groups, and that the heterogeneity of the groups is caused by misidentification, contamination or accidental swapping of samples, DNA extracts, PCR products or sequence data. We set out to test the hypothesis that phylogenetic analyses of combined morphological and molecular sequence data sets yield significantly increased levels of resolution and support compared to analyses of molecular data alone. Our results show that the hypothesis has been verified for resolution and proven with regard to support. In recent studies it is demonstrated that even stable macro– and microcharacters observed in various collections over the years, which would normally support a rather narrow species concept are not supported by molecular data. It is postulated that additional species in the genus *Psilocybe* will be identified and possibly segregated from that genus. This investigation is initiated to elucidate taxonomic and molecular status of the 'species' of Australia, especially regarding the presence of medicinally useful compounds. The term ‘species hypothesis’ (SH) for the discovered taxa in clustering on different similarity thresholds is discussed as well (Kõljalg et al. 2013).

The usage of several liberated sets of characters such as chromosomal data, morphology, geographical range, host preference, chemistry and cross validation using inferences as of multiple observed operative principles and using multiple independent arrangements of records enclosing morphology and genetic information (multiple sequences from the same specimen) launch a robust hypothesis of species boundaries (Leavitt, Moreau & Thorsten Lumbsch 2015).

A measure of the evidential support for one hypothesis over the other is Likelihood Ratio. Likelihood is the probability that the data would have arisen under the hypothesis. The likelihood ratio of the mentioned hypothesis is in the highest level.

8 Theory

There is alignment between morphology and molecular profile in a taxon (or in taxa).

9 Aim of the project

Procedures and understandings developed in this project will be useful and potentially set a benchmark for Ethnomycological studies. This study will allow for a direct molecular comparison within Australian *Psilocybe* taxa and will identify potential candidates for further investigation and study. My study, with the aim of characterizing the taxa through morphological and molecular means, will allow for cross-referenced comparisons to be made with existing described species. It will also allow for a ready and systematic means of identification of described and presently undescribed entities in Australia. The study establishes some concordance between the local medicinal applications of the fungi investigated and their constituent phytochemical groups which are relevant to the pharmaceutical industry.

(Stamets & Weil 1996) found that there are unidentified species in the genus *Psilocybe* in Australia and that some of these species may be better placed in a new genus. These unidentified species have not been investigated either from a taxonomic or pharmacological point of view. In fact, the new mechanised world syndrome has drawn the attentions how to overcome mental disorders like Depression, stress, OCD (obsessive compulsive disorder) and so on from low levels to highly intensified stage. I believe our nature contains all the constituents to suppress human medical issues. The Exotic Mushrooms and their components have always been the area of my interest. Looking back to history, wild mushrooms have been adopted in traditional medicines to cure different types of human medical problems. I believe in the state that, I live, there are unknown exotic mushrooms that harbour unique psychoactive compounds which can possibly be used to mitigate psychological illnesses in human. The wild mushrooms and traditional medicine are two hands in hands area, which then leads into the 'modern' world use of the mushrooms in new pharmaceutical era.

For both perspectives, extracted genome from different samples will be amplified in different loci by PCR and then sequencing data will be achieved by using MiSeq (Next Gene Sequencing) in this project. The data analysis of obtained

sequences by using special software (Geneious) will determine the approach of the project at the end.

The aim of this study is to assess potential candidate gene regions and corresponding universal primer pairs as secondary DNA barcodes for the fungal kingdom additional to ITS rDNA as primary barcode. The necessity for an additional marker depends on the intended purpose of an investigation. In taxa with low ITS interspecific inconsistency. The objective of this study is a molecular method for differentiation and detection of Polygenetic study.

The current work describes a morphology identification followed by molecular biology method applied to the identification of *Psilocybe* spp, which if improved, could become a valuable objective tool in the classification. In this thesis, my goal is to explore the consequences of re-formulating mushroom species identities based on taxonomy and biogeography. Therefore, aim of the present study is not only to describe a new taxon of the world mycoflora but also to introduce a potent therapeutic agent that could be explored for food and pharmaceutical purposes. However, isolation of active components and *in vivo* studies need to be designed further. Importantly, identification of previously unknown taxa and clearer elucidation of existing taxa of *Psilocybe* and closely related species will contribute to the overall documentation of the naturally occurring and introduced species of members of the Fungal Kingdom in Australia. It would appear that this study represents the first comprehensive investigation into the classification of the genus *Psilocybe* in Australia. As such, this study into the classification and characterisation of the *Psilocybe* in Australia represents a major contribution to the overall understanding of the genus worldwide. The specific techniques used in this study are not in themselves novel but their use in combined is unique in the field of taxonomy and sets a precedent for use in other genera of fungi and indeed a wider array of organisms, particularly plants. Taxonomy of this group is of international interest and sorting out species limits will be a major contribution to knowledge and statement of significance.

Our goal is to discover the potential parts of the gene as substitute molecular barcodes for the study of fungal diversity and ecology in environmental DNA samples (Větrovský et al. 2016). My study, with the aim of characterizing the taxa

through morphological and genetic means, will allow for cross-referenced comparisons to be made with existing described species. The aim of this thesis is to use molecular techniques to understand the taxonomy of species of *Psilocybe* from Australia, thereby creating a foundation for investigation of their pharmacology.

10 Method

10.1 Supplementary Materials

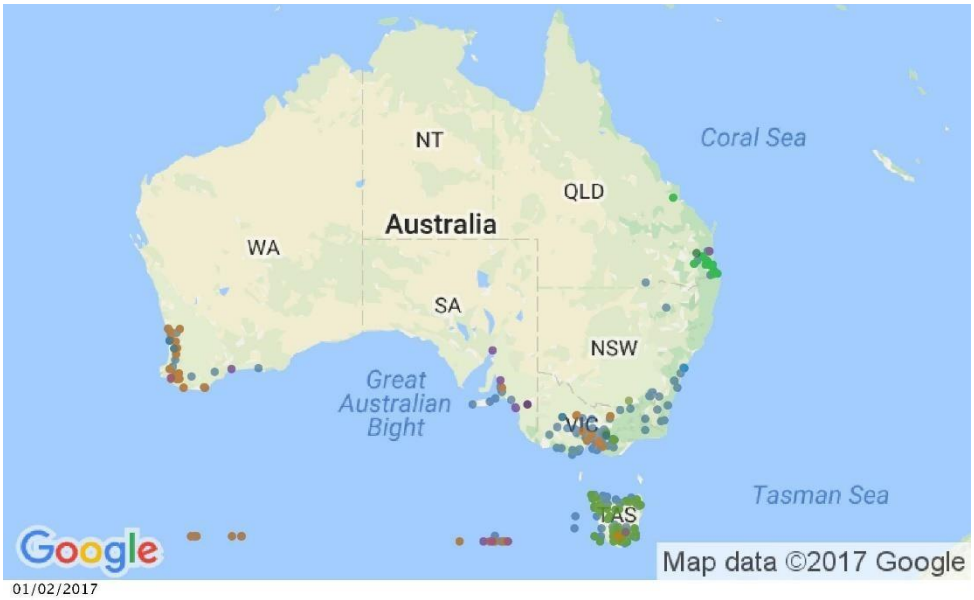


Figure 10.1*Psilocybe spp.* distribution in Australia
(Australian Virtual Herbarium website) (http://avh.chah.org.au/)

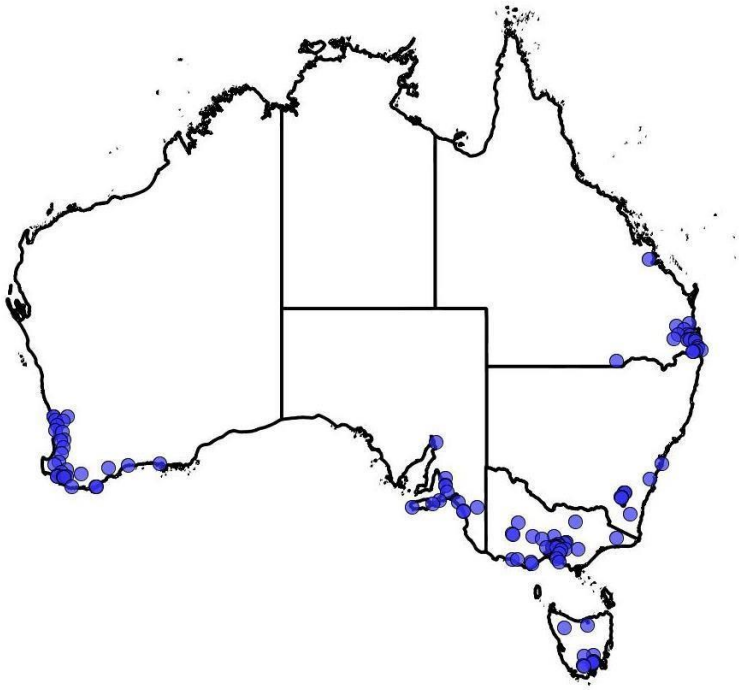


Diagram 10.1 *Psilocybe* spp. distribution in Australia
(Australia Atlas
http://spatial.ala.org.au/?ss=17F347BB16396B55F9B0A7864949A952)

10.2 Sampling

Purposive sampling has been used through the years and is currently actively employed in ethnobotany (Tongco 2007). In choosing a sampling method for informant selection, the question, the researcher is interested in answering, is of utmost importance. The identification by morphology and genetics contents of the samples will, by default, involve handling of chemically active materials. The first consideration is whether to study the entire population and if not, how to sample the population efficiently. This is possible by collecting these unidentified species from nature or check the national herbarium (Virtual Herbarium website (avh.chah.org.au/) supplemented by experts with local mycological expertise such as the 'Australasian Mycological Society' (www.australasianmycologicalsociety.com/#). As pointed out by many researchers, numerous specimens stored in herbaria are great source of genetic materials. Moreover, herbarium can possibly host a large number of presently unknown or unrepresented lineages. 70% of the taxonomic diversity in herbarium is not yet represented in the public database (Hosaka, K & Uno 2013). DNA barcoding of herbarium specimens and culture collections is extremely valuable to link unidentified sequences to known taxa (Brock, Döring & Bidartondo 2009, Nagy et al. 2011, Osmundson et al. 2013, Garnica et al. 2016). For DNA extraction, herbarium specimens are best if less than 3 or more than 15 years old.

DNA sequences have been generated from fungal type specimens > 200 years old (Larsson & Jacobsson 2004), but in many cases obtaining sequences from historical material is challenging (Dentinger, Margaritescu & Moncalvo 2010). For DNA isolation from fresh mushrooms (2m × 2m samples descent part of tissue), cells are harvested from 3–6-week-old cultures grown on cellophane sheets covering 2% malt extract agar and are lyophilized after harvest (Boekhout, Toen et al. 2002). Fresh samples can be air dried at either 20°C or 60°C (Laussmann & Meier-Giebing 2010). Freeze dried samples sealed in plastic show no detectable loss of psilocybin or psilocin when stored at -5°C or -60°C but some freeze-dried samples lose both psilocybin and psilocin over periods of one to two years when stored at room temperature. When RNA is active, it should be

stopped by freezing. The psilocybin and psilocin are fairly evenly distributed throughout the mushrooms thought the cap sometimes are more potent (Matsushima et al. 2009). In herbarium samples weight is not important. There are several potential ways that DNA can be extracted and analysed and these methods will need to be compared to clearly identify those methods that are most useful to the molecular profiles of the species being studied (Ramírez-Cruz et al. 2013). The series of experiments is carried out with living fungi and their extracts which is derived from voucher specimens (Voucher specimen is a preserved specimen that serves as a verifiable and permanent record of wildlife as it preserves as much of the physical remains of an organism as possible). Vouchered specimens are photographed and dried for future study.

Authors in the field have suggested different ways to obtain extracts from mushrooms. Whatever the source of information, it is important that the author refer to voucher samples lodged in a herbarium. These vouchers can be used to support the identification of the species (Lukhoba, Simmonds & Paton 2006).

Voucher specimens of the cited fungi species are prepared and deposited. Once the species of voucher specimens have been determined, or if possible, their prior determination confirmed, compilation and organization by taxa, vernacular scientific names, use categories and other groupings provide enormously valuable data for direct utilization or comparison with different data sets.

In many studies carried out by workers it has been frequently reported that alcoholic extracts generally show greater activity as compared to other solvents like water, petroleum, ether etc. This may be due to the nature of the alcohols to dissolve the organic compounds better than water (Singh et al. 2011). In case of negative results because of any reason they are re-examined after 5-6 weeks (Boekhout, Toen et al. 2002). When maintaining specimens for future molecular examination, we use 95–100% ethanol. Concentrations of 90% may be adequate to preserve material when directing rDNA and mtDNA with hundreds of replicas per cell; however, it may render samples unusable for future amplification of single copy markers or for the application of novel DNA technologies that require well-preserved, high-quality DNA for future study (Blasco-Costa et al. 2016).

A subset of 96 *Psilocybe* specimens is selected and examined. This number address 10% of the total number out of global herbarium specimens and 15% of the total number of Australia and New Zealand herbarium specimens. (The total number of herbarium specimens in biggest European herbarium, Kew in UK, is 6 and for New York botanic garden herbarium this number is 422. The total number of herbarium specimens for Australia and New Zealand is 674). These numbers show the exclusiveness of our sampling (Table 10.1.).

Table 10.1 MEL_Psilocybe_Continue.xls

10.3 General Morphology

Morphological Species Concepts (MSC) defines species as groups of individuals that are morphologically similar to one another and are morphologically distinct from other such groups. Standard morphological identification methods are used for differentiation and classification of the various 'species' of all mushrooms presently placed in the genus *Psilocybe* and a few presumed closely related taxa (Keller et al. 1999b, Lee, Cole & Linacre 2000a). However, this genus could not be identified morphologically because of several unclear phenotypic characters. Many of the taxonomic keys to identify fungi are based primarily on morphological criteria. Fungi in the first three quarters of the 20th century are originally identified incorrectly by one of several oversimplified morphological structures (Cheng et al. 2008).

The first pass of the morphological measurements was done on herbarium's specimens. The dried specimens were analysed using macro and micro morphological characteristics that follow formerly designated techniques (Rossato et al. 2009). The macro morphological characteristics were recorded on the dried samples and from notes attached to herbarium specimens. Macroscopic characters were recorded from at least 4-8 fruiting bodies of each collection, covering all stages of maturity. Delineation of special characters followed by the book of 'PSILOCYBIN MUSHROOMS of the WORLD' (Stamets &

Weil 1996) which completely explained all potential characters of these fungi with emphasise of the most important ones.

The microscope-based information was gained from the dried specimens using standard procedures. Microscopic characters were recorded from dried material rehydrated in 3% aqueous KOH (w/v) with 0.5% aqueous Congo Red (w/v) (Stefani, Jones & May 2014). Present identification of fungi is based on morphological characteristics of the mushrooms (fruiting bodies) and observation of a combination of the macroscopic and microscopic characteristics. These features include general shape and size, gill constructions and specific colour, size and patterning of the spores. These characteristics are readily observable when the fungi are in a reasonable condition (Linacre, A, Cole & Lee 2002). Spores and Basidia is taken from lamellae fragments of mature fruit bodies. Twenty spores were measured from one to two fruit-bodies of each collection. We mounted the spores in Melzer's reagent and examined the decoration of spores by using Scanning Electron Microscopy (SEM) (Stefani et al., 2014).

Additional identification techniques to apply to the genus *Psilocybe* is utilised for primary identification of herbarium specimens. These genus specific methods include: spore prints, bluing reactions (just for fresh samples) and the existing combined dichotomous key for identification. A standard method of species identification of fungi is morphological analysis, which includes macroscopic and microscopic investigations. The identification of the fungi themselves is used upon reflection of a combination of the macroscopic and microscopic individualities. These later comprise the gill arrangements and spores (Linacre, A, Cole & Lee 2002).

10.3.1 Macroscopic studies

Description*:* Mushroom is an ambiguous and common term used to define the fruit bodies of fungi mainly all gill fungi which have typical cystidia and basidia (Tsujikawa et al. 2003).

Macroscopic analysis involves in determining the colour, size and characteristic structures of mushrooms. Characteristic features of stipe such as stipe length,

colour, texture, density and configuration should be recorded. Characteristic features of Pileus such as size in diameter, colour, texture, shape and edge (margin) should be examined and recorded. Pileus and Stipe should be bruised to record any changes of colour (Smith & Weber 1980). Annulus and veil should be observed to note their presence or absences and recorded. Lamellae should be also observed to note their colour, attachment to pileus, texture, and edge using magnifying lens.

10.3.2 Microscopic studies

The prolonged and laborious microscopic examination is principally based on a comparison of the appearance of spores, which does not always yield satisfactory fallouts (Zuber, Kowalczyk, Sekula, et al. 2011). Microscopic features are found from dried material by mounting free-hand sections of basidiocarps rehydrated with 96% alcohol and then mounted in 3% aqueous KOH, Meltzer's reagent, 1% Congo Red and ammonia solution (Dutta et al. 2015). The specimens are photographed in situ collected and then analysed macro and micro-morphologically (Rossato et al. 2009). A minimum of 20 microstructures (basidia, basidiospores, cystidia and hyphae) are observed which are drawn with the aid of a light tube at magnification of 1000x. The studied herbarium resources are observed by light microscopy through slides mounted in 3% KOH solution (Guzmán, G & Kasuya 2004).

Spores free of tissue can be obtained from the sample simply by washing the gills in the 10% ammonia. Melzer's reagent when placed on tissues or spores might turn them blue or bluish-black purple- brown or golden to yellowish. The colour alteration in characteristic for the fungi under study. If they convert blue, bluish-grey or blue-black they are amyloid and if they are unaffected or only become somewhat yellowish, they are turned inamyloid or non-amyloid. Mushrooms of the genus *Psilocybe* are constantly amyloid (Musshoff, Madea & Beike 2000). Basidiospore dimensions measurements indicate length and width of spores in face sight (face view) and maximum depth of spores (width and thickness) in profile view (side view) (Guzmán, G et al. 2003).

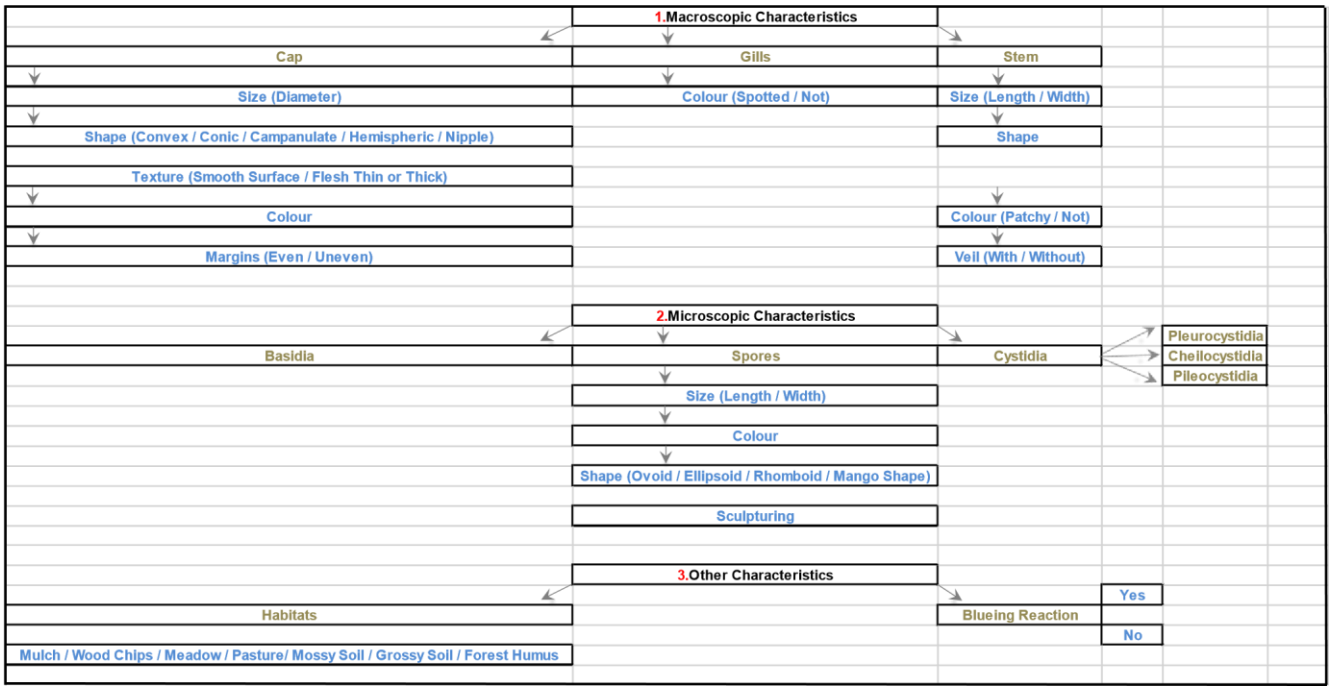
Morphologically, fungi can range from microscopic single-celled yeasts to filamentous moulds and macroscopic multicellular mushrooms (James 2015).

Micro-morphological data are obtained from the dried specimens and observed under a light microscope. Colours of the spore print should be observed and recorded. Slides should be prepared from collected spores and mounted in

Meltzer's reagent. Shapes of basidia, cystidia, arrangement and number of basidiospores on basidia should be recorded (Al-Momany & Saleh 2009). Fungal tissues or gills from the mushrooms are removed with a sharp scalpel, stained with Meltzer's reagent, mounted on slides and slightly tipped onto coverslips. The slides are observed under the microscope and measured and imaged (Wang & Tzean 2015). After recording the morphological features of the collected mushrooms, they should be compared with documented species using the keys to ascertain their identity (Musa, Wuyep & Gbem).

For microscopic analysis, the dry materials are rehydrated in 70% ethanol. Microscopic characters are observed in thin sections of dry basidiomata mounted in 3% KOH, 0.1% (w/v), Congo-Red, Melzer's reagent (Dring 1971) or 0.1% (w/v) cotton blue in lactic acid. Qualitative characteristics such as the colour and shape of the pileus, colour of stipes, and colour of mushroom spore print are evaluated by eye at first stage. For microscopic characteristics, freehand transverse sections 0.1 mm thick are made from rehydrated basidiocarps using a sharp surgical blade. The sections are immersed in a diluted solution of methyl blue stain and left for 10 min. The thinnest sections are selected, placed on glass slides and covered with cover slips. Low-power (×40) objectives of a standard light microscope are used to observe the basidia in the sections; colours and sizes of the basidiospores are also should be determined. Basidia and basidiospore size are measured from 10 basidia and 20 basidiospores each (Table 10.2.).

Table 10.2 Different main Macro and Micro characteristics of *Psilocybe* fungi



Taxonomic ambiguities between these taxa (*Psilocybe*) have been originated from traditional classification which is unclear due to similar morphological and ecological features. Therefore, not only morphological characters but also molecular data are needed to identify this macro-fungus (*Psilocybe*) correctly. To date, morphological analysis has been the keystone to phylogeny systematics. However, since the late-1980s we have seen an increased combination of genetic data to overcome difficulties faced when morphological data are considered in isolation (Blasco-Costa et al. 2016). The weakness of morphology is that species identified by morphology frequently include more than one species when detected by phylogeny or biology (Taylor et al. 2000). The lack of distinctive morphological characteristics means that morphology itself does not provide an adequate basis for recognition of a new taxon. Some of these distinctions have been confirmed using molecular data, it means that molecular data may be useful in determining if clear distinctions can be made. Many species classified in sub-genus are morphologically similar and identification, using traditional morphological techniques remains difficult. Some species are difficult to identify because of a lack of clear-cut morphological criteria to

discriminate not only among species within the genus but also among the closely related genera. This has resulted in numerous taxonomic problems

in the naming of species with the result that species have often been placed in several closely related genera. Traditional morphology-based taxonomy is critical to this effort. In most of the species, morphological differences are essentially non-existent. Differences in morphology are nebulous and a matter of degree rather than sharply defined. There is similarity of morphology among species level. Thus, as widespread as is morphology and as straightforward as it is to apply, it cannot be reckoned to diagnose evolutionarily meaningful species in fungi (Taylor et al. 2000).

Though identification of the genus in the field is not difficult, determination at the species level can be confusing because of similarities in morphological characters. Key features are; photograph, colour, stalk length, stalk diameter, and cap diameter. The important data is considered such as size, shape and ornamentation of the fruiting body and basidiospores. Many of the isolates attained by traditional techniques can simply be identified to mycelia sterilia and are often grouped as `morphospecies' based on comparable cultural characters. There are being many species that appear to be similar under cultural conditions and exhibit similar morphology, but are in fact different species (Jeewon & Hyde 2007). Arrangement of taxa into morphospecies, however, does not reflect species phylogeny, and hence alternative approaches are required for the identification of designated morphospecies for further documentation (Guo, Hyde & Liew 2000). Optimised morphological method based on the important characters (Spore and Basidia), help this research to be more reliable. This method can be an anchor for the modern morphology-based studies too. This way of thinking for readjustment the traditional morphology route provides the strong tools as a foundation for new discovered method of classification.

Morphological characters are still beneficial for primary estimation since it is fast, simple, and can be used as an overall approach for calculating genetic diversity among morphological distinguishable accessions (Inyod et al. 2017). The morphological and biochemical identifications of fungi sometimes face many problems such as: the need for a great time, requiring high skill, and generating

various morpho / biotypes within one species. Identification of fungal species based on morphology alone is often unreliable and problematic to apply due to the absence of a strong morphological distinction between carefully connected species and the wide morphological deviation among genera and sometimes even families. Classification system of organisms are historically based on observable characteristics. Organisms, including fungi, have traditionally been classified based on morphological characteristics, although recently genetic analysis has been used to determine some higher-level taxa. For a long time, phenotypic procedures including morphology, physiology, and biochemistry were the base of taxonomy and identification of fungi, but these are time-consuming, inaccurate, and in many cases inefficiently conclusive to classify the less-common taxa or isolates theoretically demonstrating novel species (Mirhendi et al. 2015).

Morphological characters (Smell, taste, spore ornamentation) currently represent the basis for classification for field mycologists, as well as ecological factors (geographical origin, habitat), but genetic analysis should be carried out in case of doubtful or inconclusive findings, and should be especially requested when new taxa are described. Morphological information has been shown to be of limited value for fungal systematic due to their natural simplicity, evolutionary convergence, parallelisms, and phenotypic flexibility (Hussein et al. 2014). However, the division between intraspecific phenotypic plasticity and interspecific deviation can become somewhat masked. Even more interesting, when using morphological data in isolation is the identification and equivalent of the life-cycle stages, freely reachable DNA sequencing technologies have provided taxonomists with a free source of data to help overcome the limits, linked with morphological analyses (Blasco-Costa et al. 2016).

This genus (*Psilocybe*) is found primarily in association with well-rotted wood and it is characterized by free lamellae, absence of a volva, pinkish spore print, inamyloid basidiospores and inverse hymenophoral trama. Most sectors of *Psilocybe* following classification, built on morphological features are not supported in phylogenetic analysis. Inherited character state reconstruction analysis suggests that basidiospore figure in anterior view and spore wall thickness as key factors. The composite of characters (bluing reaction of

basidiomata, pileus figure, existence and sort of annulus, growing substrate, form, colour and wall thickness of basidiospores, content colour of pleurocystidia and cheilocystidia) used to delineate the species, is selected in order to cover morphology (Ramírez-Cruz et al. 2013).

Macroscopic characters such as basidiospores with a coloured endosporium ornamented with columns or crests, and a hyaline smooth exosporium, pileus striate, peeling pellicle, veil on pileus, lamella colour, veil on stipe, spore length, spore width, spore breadth, spore f/l index, spore wall, spore wall colour, germ pore, pleurocystidia, pileal surface, presence or absence of veil cystidia (Boekhout, Toen et al. 2002), basidiospore wall: thin walled, faintly thick walled, thick walled, can be considered (Ramírez-Cruz et al. 2013). All spores of *Psilocybe* mushrooms are of similar shape, brownish and smooth (Musshoff, Madea & Beike 2000).

The core of the genus *Psilocybe* contains many species with specialized terminal cells (Cystidia) that occur on the basidiomata (fruit-bodies) and are often thick-walled and apically incrusted with precipitates of calcium-oxalate (Abadio et al. 2012). Pileus 25–70 mm broad, up to 15 mm high, convex campanulate to subumbonate, Plano-convex with only an obtuse umbo, smooth, young cream or buff, ochraceous towards the umbo, then brownish red, with white spots, margin with whitish veil fragments, becoming patchily blueish. Lamellae adnate or adnexed, slightly crowded, ochraceous at first, then greyish violet to dark violet brown, with whitish subflocculose edges. Stipe 50– 105 35–15 mm, sub-equal tapered upwards from a most distinctly swollen base, hollow, white to pale yellowish, with a white, persistent but fragile annulus, smooth to subflocculose, blueing. Context whitish, blueing when cut, no distinct smell recorded. Spores 9.5–11.53 5.5–7.0 µm, sub-hexagonal in face view, (sub-) ellipsoid in side view, thick walled, with a distinct broad apical germ pore, dark yellowish brown. Basidia 4-spored, clavate cylindric. Cheilocystidia numerous, thin walled, hyaline, fusiform to sub lageniform, up to 30 µm long. Pleurocystidia scarce, similar to Cheilocystidia. Cap cuticle of narrow, radially arranged, gelatinized, cylindric, and hyaline to yellowish hyphae, clamp connections present (Keller et al. 1999a).

The morphology of the young fruiting body (Basidiomycotina and within the Polyporales) has a circular cap incurved or enrolled inside. Then, the mature fruiting body has a wider cap than the young. The young fruiting body has a pattern of universal veil remnant (scabrous) on the cap surface, but in mature fruit bodies, it may be absent or remain as scales on the cap surface. Mushroom cap shapes are central depression (young fruiting body) or infundibuliform (mature fruiting body). Mushroom caps are ornamented and the entire cap surface and margin. The stem is erected from bases. Mushroom gills are adnexed (young fruiting body) or decurrent (mature fruiting body) of attachment to the stem, crowded of gill spacing, and smooth (young fruiting body) or wavy (mature fruiting body) of gill margins. Mushroom stems are equal in stem shape, bulbous of stem base, free of the membranous annulus, and fragmented bands on the stem surface. Using an optical microscope (Motic BA200) under magnification up to 1500, they are classified into groups based on the diameter of the pileus and their stipe length ratio (INYOD et al. 2017).

<u>One example of fully explained characteristic Morphology (Macroscopy/Microscopy) Descriptions (Sample No.2363659)</u>

Pileus 18 × 9 mm, convex or slightly umbonate with a small obtuse umbo, viscid, trans. st. at margins, brown. Margin is straight and entire. Lamellae decurrent, arcuate, deep to 6 mm, very dark brown, close, thin, lamellulae. Stipe 30 × 2.5 mm or 23 × 2 mm, centrally attached, flexuous, wiry, tough, fibrous, reddish brown, blackening at base. Odour: acrid

Spores 6 × 8.5 (11.5) µm, Q = 1.41 (1.91)

Basidia 4-spored 19 × 8.5 µm

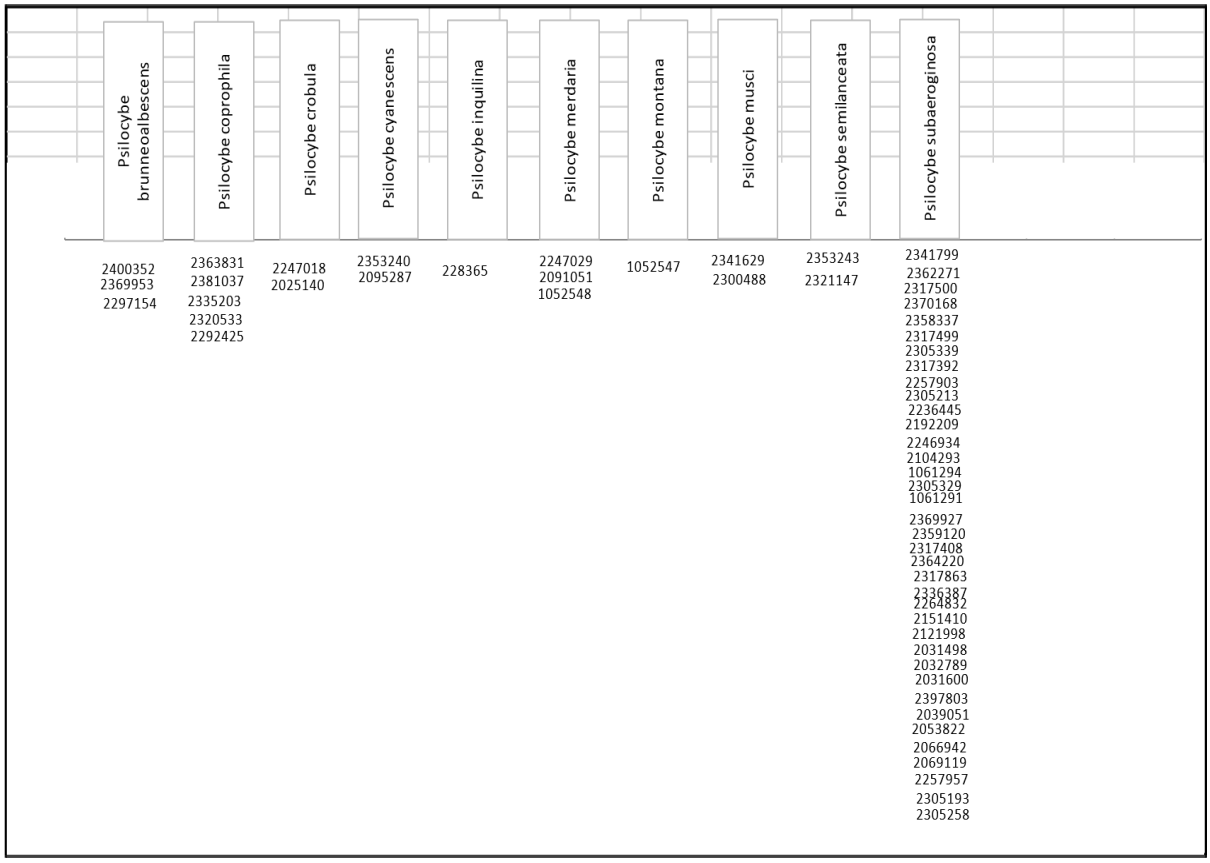
Cheilocystidia 29 × 6 µm

Habitat: Wet sclerophyll forest severely burnt

Substrate: Soil

<u>Visualised morphology statistics:</u> (Tables 10.3-10.19, Diagrams 10.2-10.17)

Table 10.3 Morphologically named *Psilocybe* fungi in Melbourne Herbarium



<u>Morphological characters of named and unnamed *Psilocybe spp.* of Melbourne herbarium</u> (Tables 10.4., 10.5., 10.6.)

Table 10.4 Morphology Data 1.xlsx
Table 10.5 Morphology Data 2.xlsx
Table 10.6 Morphology Data 3.xlsx

Table 10.7 Morphologically named *Psilocybe* from Melbourne herbarium

Melbourne Named *Psilocybe* Species	Grade	*Genus & Spp*	code
2257903	1.00	*Psilocybe subaeroginosa*	10
2236445	1.03	*Psilocybe subaeroginosa*	10
2358337	1.05	*Psilocybe subaeroginosa*	10
2370168	1.07	*Psilocybe subaeroginosa*	10
2246934	1.09	*Psilocybe subaeroginosa*	10
2257957	1.11	*Psilocybe subaeroginosa*	12
2069119	1.13	*Psilocybe subaeroginosa*	12
2066942	1.15	*Psilocybe subaeroginosa*	12
2053822	1.17	*Psilocybe subaeroginosa*	12
2305193	1.19	*Psilocybe subaeroginosa*	12
2305339	1.21	*Psilocybe subaeroginosa*	10
2362271	1.23	*Psilocybe subaeroginosa*	10
2397803	1.25	*Psilocybe subaeroginosa*	12
2317392	1.27	*Psilocybe subaeroginosa*	10
2192209	1.29	*Psilocybe subaeroginosa*	10
1061294	1.31	*Psilocybe subaeroginosa*	10
2317499	1.33	*Psilocybe subaeroginosa*	10
2317500	1.35	*Psilocybe subaeroginosa*	10
2305213	1.37	*Psilocybe subaeroginosa*	10
2341799	1.39	*Psilocybe subaeroginosa*	10
2039051	1.41	*Psilocybe subaeroginosa*	12
2305329	1.43	*Psilocybe subaeroginosa*	10
2305258	1.45	*Psilocybe subaeroginosa*	12
2104293	1.47	*Psilocybe subaeroginosa*	10
2151410	1.49	*Psilocybe subaeroginosa*	11
2264832	1.51	*Psilocybe subaeroginosa*	11
2317863	1.53	*Psilocybe subaeroginosa*	11
2359120	1.55	*Psilocybe subaeroginosa*	11
2336387	1.57	*Psilocybe subaeroginosa*	11
2369927	1.59	*Psilocybe subaeroginosa*	11
2364220	1.61	*Psilocybe subaeroginosa*	11
2031600	1.63	*Psilocybe subaeroginosa*	11
2032789	1.65	*Psilocybe subaeroginosa*	11
2031498	1.67	*Psilocybe subaeroginosa*	11
2121998	1.69	*Psilocybe subaeroginosa*	11
2317408	1.71	*Psilocybe subaeroginosa*	11
1061291	1.73	*Psilocybe subaeroginosa*	11
2353243	2.00	*Psilocybe semilanceata*	9
2321147	2.03	*Psilocybe semilanceata*	9

2300488	3.00	*Psilocybe music*	8
2341629	3.03	*Psilocybe musci*	8
1052547	4.00	*Psilocybe montana*	7
2247029	5.00	*Psilocybe merdaria*	6
1052548	5.03	*Psilocybe merdaria*	6
2091051	5.05	*Psilocybe merdaria*	6
228365	6.00	*Psilocybe inquilina*	5
2095287	7.00	*Psilocybe cyanescens*	4
2353240	7.03	*Psilocybe cyanescens*	4
2247018	8.00	*Psilocybe crobula*	3
2025140	8.03	*Psilocybe crobula*	3
2381037	9.00	*Psilocybe coprophila*	2
2363831	9.03	*Psilocybe coprophila*	2
2292425	9.05	*Psilocybe coprophila*	2
2320533	9.07	*Psilocybe coprophila*	2
2335203	9.09	*Psilocybe coprophila*	2
2297154	10.00	*Psilocybe brunneoalbescens*	1
2369953	10.03	*Psilocybe brunneoalbescens*	1
2400352	10.05	*Psilocybe brunneoalbescens*	1

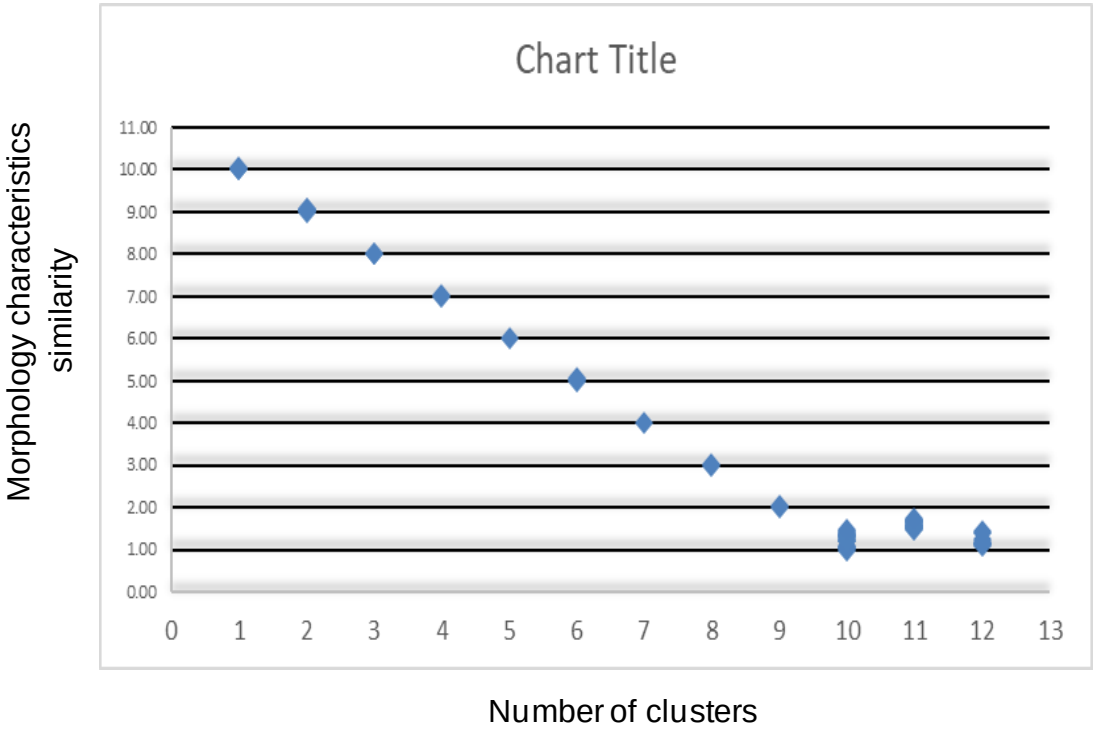


Diagram 10.2 Morphologically categorised and named *Psilocybe* dispersion from Melbourne herbarium

Table 10.8 An example of calculating Q for a sample in morphology characteristic determination

(The symbol Q is used to indicate mean “length/width ratio” of a spore in side view or basidiospores in profile)

Collection	TWM 567		
	Spore L	**Spore W**	**Q**
	10	7.5	1.33
	9	6.5	1.38
	8	4.5	1.78
	7	5	1.40
	9	6	1.50
	8	7	1.14
	9	5.5	1.64
	8	4.5	1.78
	9	5	1.80
	10	8	1.25
No. measured			**10**
Mean	**8.70**	**5.95**	**1.50**
Min	7.0	4.5	1.14
Max	10.0	8.0	1.80
Collection	MEL 675656		
	Spore L	**Spore W**	**Q**
	10	7.5	1.33
	9	6.5	1.38
	8	4	2.00
	7	5	1.40
	9	6	1.50
	8	7	1.14
	12	4.5	2.67
	9	5.5	1.64
	8	4	2.00
	9	5	1.80
	9	6	1.50
	8	7	1.14
	9	5.5	1.64
	8	4	2.00
	9	5	1.80
	7.5	7	1.07
	6.5	6	1.08
	7	5	1.40
	8	5	1.60
	10	8	1.25
No. measured			**20**
Mean	**8.55**	**5.68**	**1.57**
Min	6.5	4.0	1.07
Max	12.0	8.0	2.67

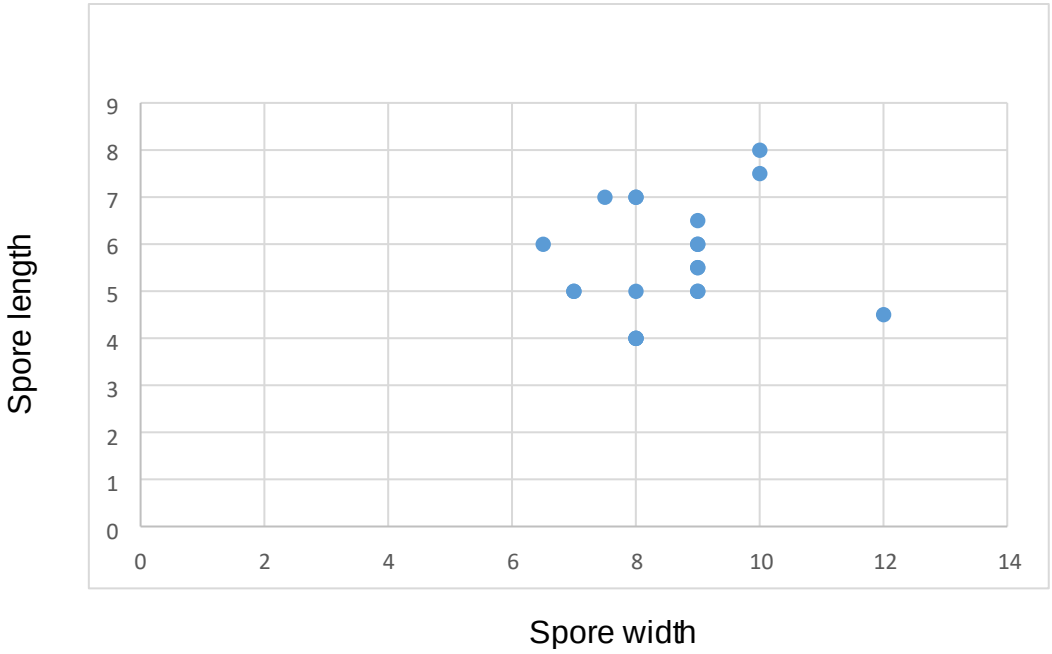


Diagram 10.3 Spore (major characteristic) size measurements

Table 10.9 Three main characters of the whole data set for 15 random samples (spores, basidia, cheilocystidia)

Melbourne Unknown *Psilocybe* Species	Spores n=20			Basidia n=10		Cheilocystidia	
	(Mean Length × Mean Width) µm		Mean Q	(Mean Length × Mean Width) µm		(Mean Length × Mean Width) µm	
2103697	16.00	9.60	1.68	22.7	14.30	30.00	14.00
2054573	7.3	3.9	1.89	28.6	9.6	25	7.5
2025255	13.25	9.1	1.46	27.7	11	29.25	11.25
2322085	11.4	6.7	1.71	26.5	8.9	29.4	12
2112647	11.23	6.35	1.78	32.1	8.65	31	8.5
2367138	8.15	5.15	1.61	24.6	7.05	31.6	6.2
2367327	12.43	7.95	1.58	31.7	10.6	47.5	10.5
2367329	9.05	5.48	1.65	20.8	8.7	28.5	6
2358338	8	4.4	1.84	23.9	7.8	37.6	5.6
2363659	9.95	6.2	1.61	19.5	8.3	29	6
269073	13.35	7.6	1.76	29.9	11.8	30	7
269070	8.03	5.43	1.48	26	8	27.5	6.75
2317471	9.55	5.68	1.69	20.3	7.7	26.7	6
2335174	8.3	4.95	1.71	24	24	24	3.5
2321029	14.4	7.85	1.84	28.6	28.6	60	10

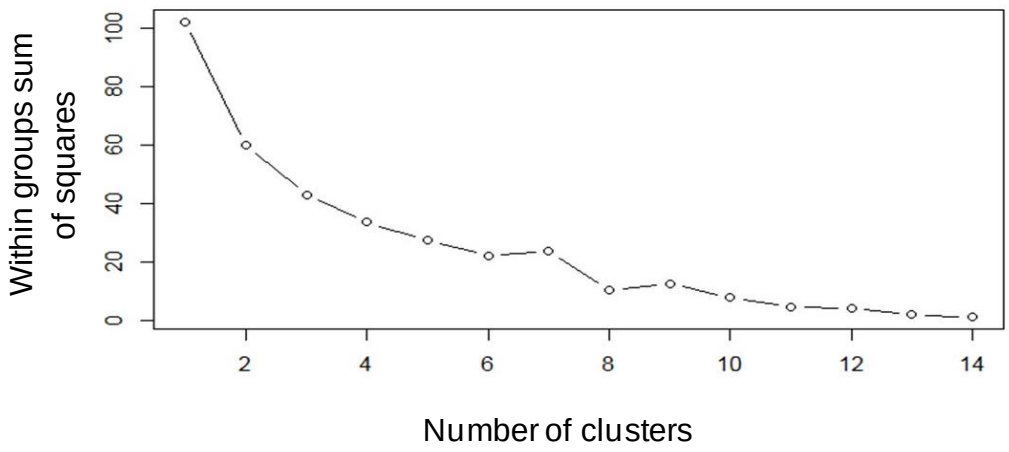


Diagram 10.4 Clustering based on spores, basidia and cheilocystidia dimensions (seven clusters)

Table 10.10 Category clustering table based on three main characters (spores, basidia, cheilocystidia) of the whole data set for 15 random samples

Melbourne Unknown *Psilocybe* Species	**cluster**
2367138	1
269070	1
2321029	2
2335174	3
2103697	4
2025255	4
2322085	5
2112647	5
2367327	5
269073	5
2054573	6
2358338	6
2367329	7
2363659	7
2317471	7

Diagram 10.5 (a-b-c) 7 clusters of three characteristics (spores, basidia, cheilocystidia) for 15 random samples

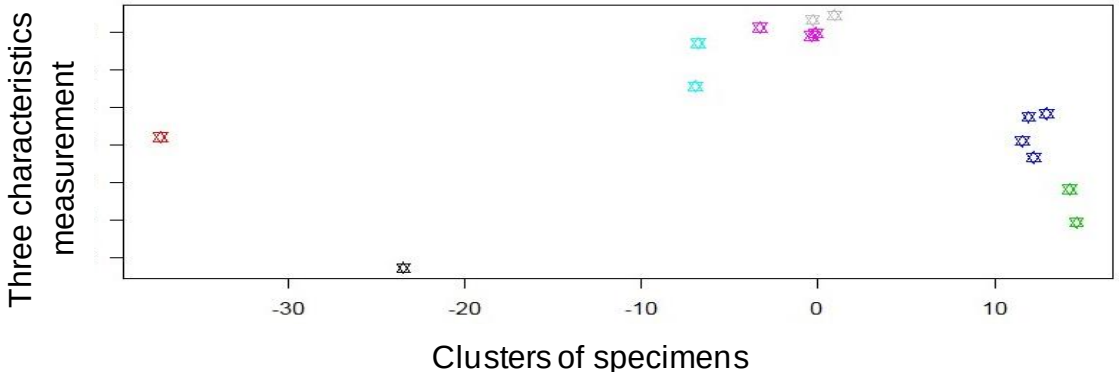


Diagram 10.5a. Seven clusters of three characteristics (spores, basidia, cheilocystidia) for 15 random samples

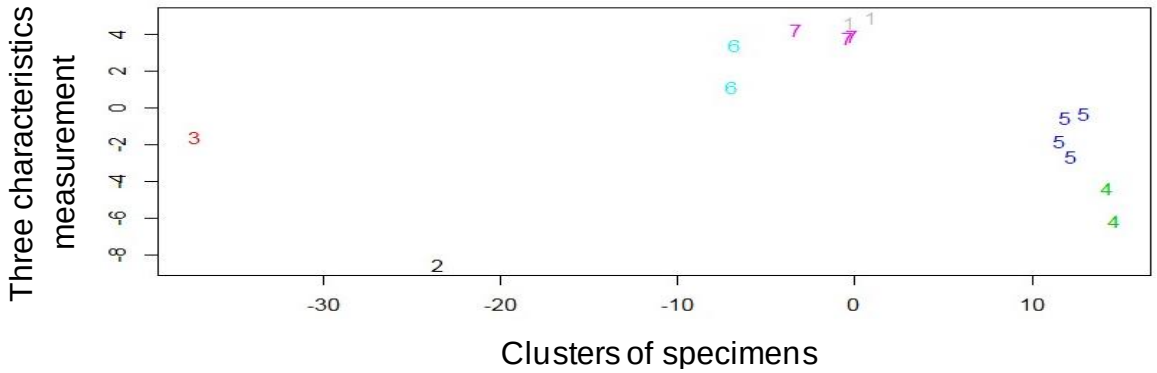


Diagram 10.5b. Seven clusters of three characteristics (spores, basidia, cheilocystidia) for 15 random samples

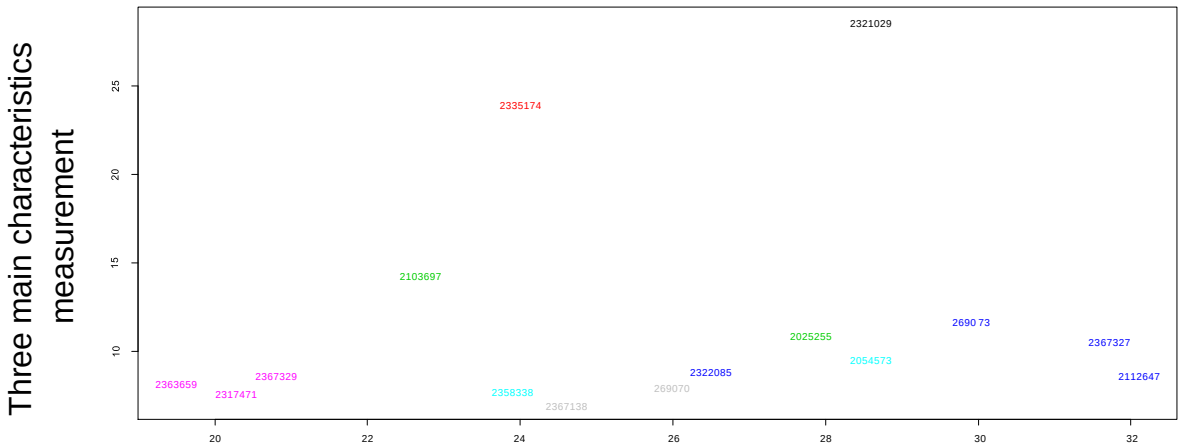


Diagram 10.5c. Seven clusters of three characteristics (spores, basidia, cheilocystidia) for 15 random samples

Table 10.11 Number of clusters from two characters pileus and stipe for nine random samples

Melbourne Unknown *Psilocybe* Species	Pileus	Stipe 1	Stipe 1
2367138	20	26	2.5
2367327	18	40	2
2367329	10	30	1
2358338	15	20	2
2363659	18	30	2
2358340	17	15	1
228403	25	80	3
2317471	8	20	1
2335646	20	40	2

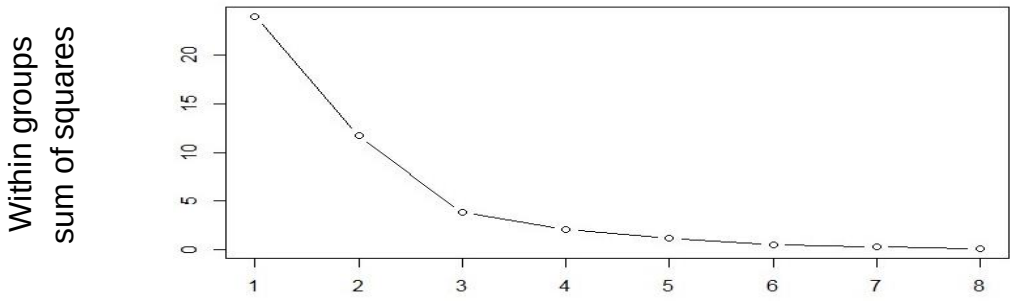


Diagram 10.6 Clustering for two characters; Pileus and Stipe

Table 10.12 Category table of clusters from two characters; pileus and stipe for nine samples

Melbourne Unknown *Psilocybe* Species	cluster
2358340	1
2367329	2
2317471	2
228403	3
2367138	4
2367327	4
2358338	4
2363659	4
2335646	4

Diagrams 10.7 (a-b-c) 4 clusters of two characters (Pileus and stipe) for 9random samples

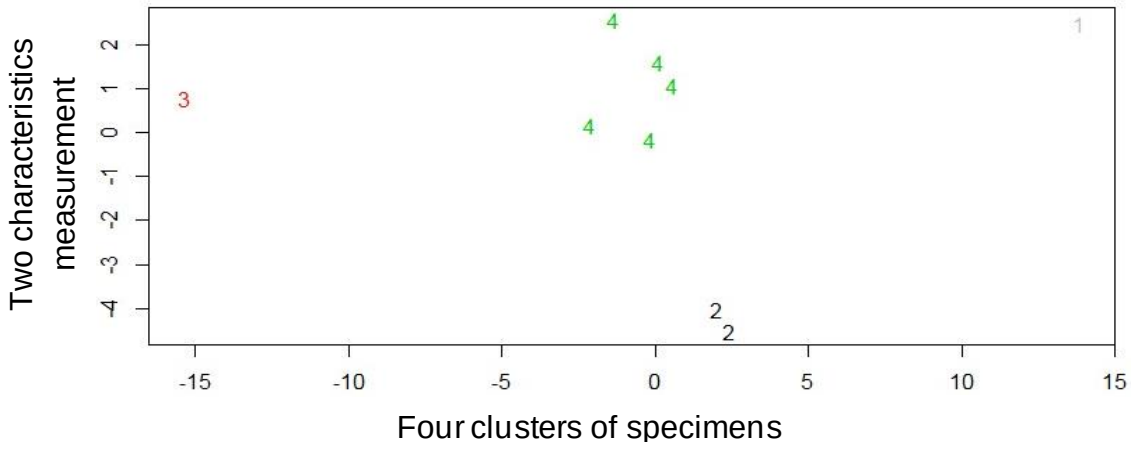


Diagram 10.7a. Four clusters of two characters; pileus and stipe for nine samples

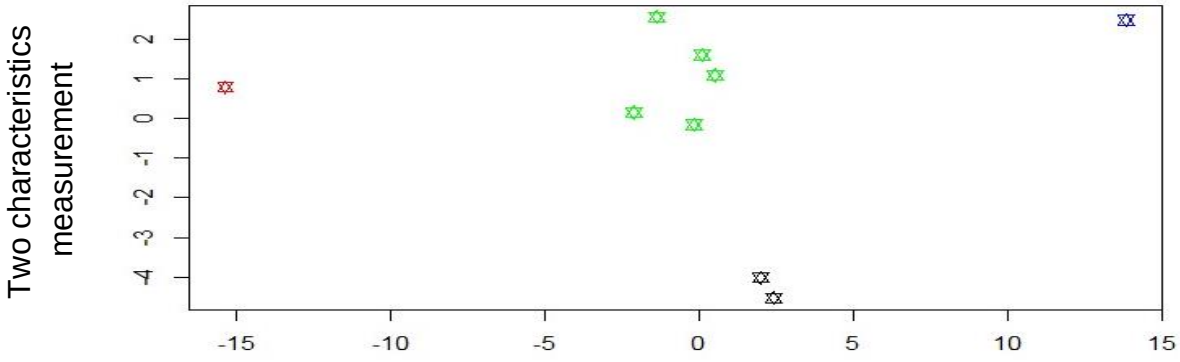


Diagram 10.7b. Four clusters of two characters; pileus and stipe for nine samples

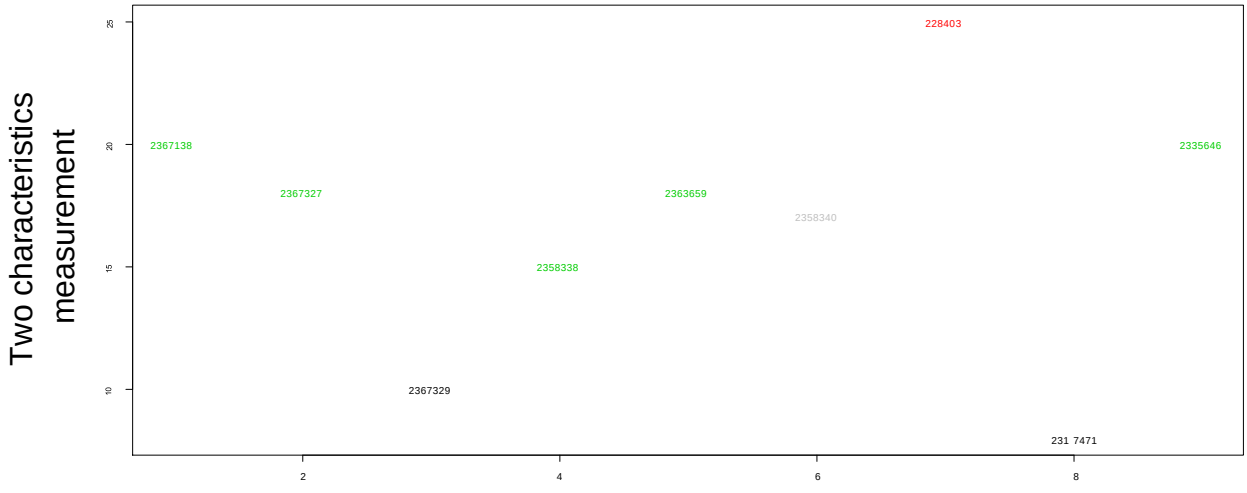


Diagram 10.7c. Four clusters of two characters; pileus and stipe for nine samples

Table 10.13 Spores measurement in two dimensions for 35 samples

Spores n=20			
Melbourne Unknown Psilocybe Species	**(Mean Length × Mean Width) µm**	**(Mean Length × Mean Width) µm**	**Mean Q**
2103829	8.2	4.9	1.68
2103697	16	9.6	1.68
2054573	7.3	3.9	1.89
2025255	13.25	9.1	1.46
2362243	6.95	4.2	1.66
2322085	11.4	6.7	1.71
2112647	11.23	6.35	1.78
2367138	8.15	5.15	1.61
2367327	12.43	7.95	1.58
2367329	9.05	5.48	1.65
2358338	8	4.4	1.84
2363659	9.95	6.2	1.61
2321893	7.15	4.1	1.75
269072	11.75	8.4	1.41
2316761	7.4	5.5	1.38
269073	13.35	7.6	1.76
1055464	7.3	5.8	1.27
269071	13.2	8.45	1.55
228403	11.55	7.65	1.51
269070	8.03	5.43	1.48
2104350	14.1	8.1	1.74
2300763	8.3	5.45	1.53
2231581	7.65	4.3	1.79
2103668	13.4	7.45	1.81
2341406	12.3	7.35	1.68
2317471	9.55	5.68	1.69
2335646	6.9	4.45	1.57
2320554	8.05	5.25	1.54
2314629	12.05	6.6	1.8
2335174	8.3	4.95	1.71
2335167	9.35	5.65	1.66
2321061	8.05	5.3	1.53
2321029	14.4	7.85	1.84
1061315	8.95	5.3	1.7
2031497	14.3	9.1	1.58

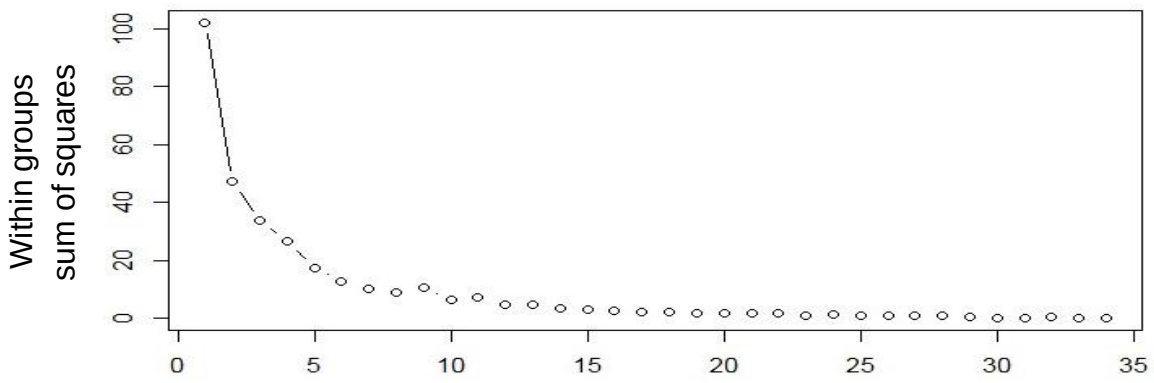


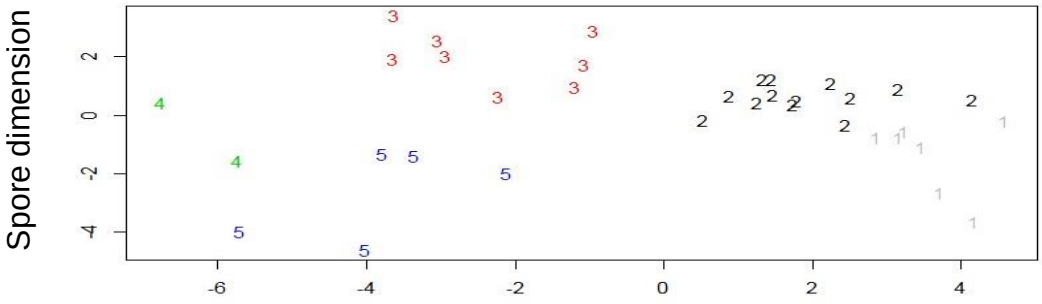
Diagram 10.8 Clustering based on dimension of spore characteristics (5 clusters)

Table 10.14 Spores dimension clustering (35 samples)

Melbourne Unknown *Psilocybe* Species	cluster
2316761	1
1055464	1
269070	1
2300763	1
2335646	1
2320554	1
2321061	1
2103829	2
2054573	2
2362243	2
2367138	2
2367329	2
2358338	2
2363659	2
2321893	2
2231581	2
2317471	2
2335174	2
2335167	2
1061315	2
2322085	3
2112647	3
269073	3
2104350	3
2103668	3
2341406	3
2314629	3
2321029	3
2103697	4

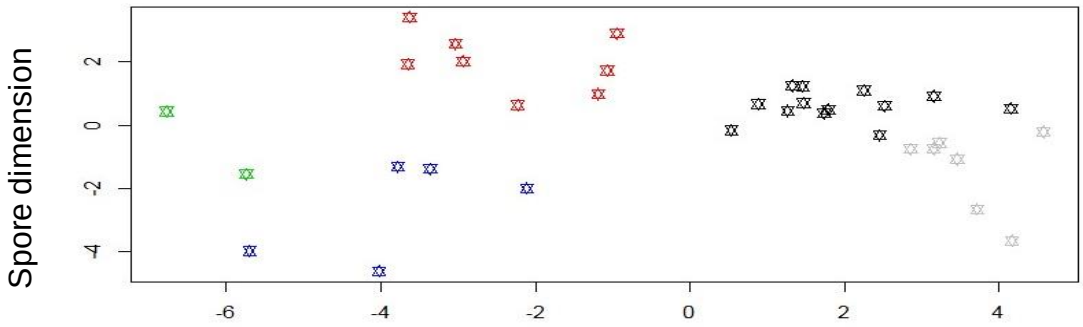
2031497	4
2025255	5
2367327	5
269072	5
269071	5
228403	5

Diagram 10.9 (a-b-c) 5 clusters of spore dimensions (35 samples)



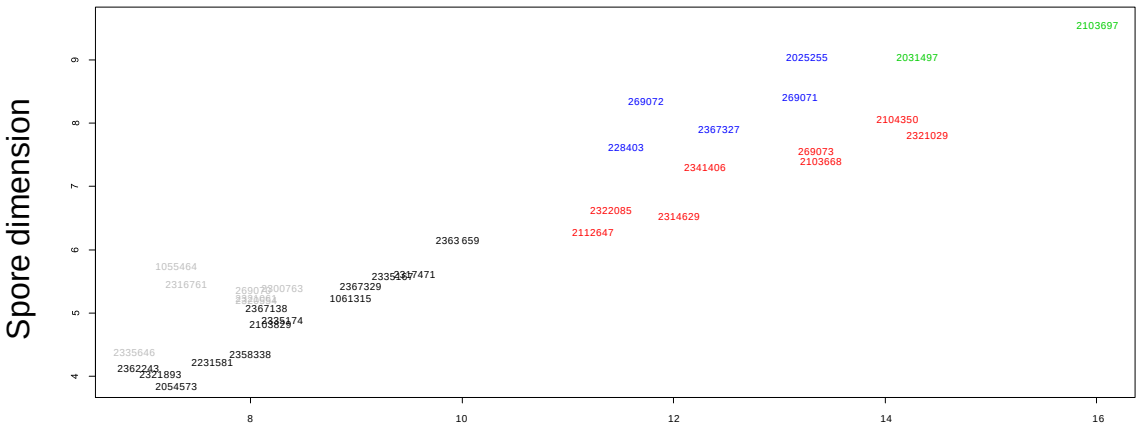
Five clusters of specimens

Diagram 10.9a. 5 clusters of spore dimensions (35 samples)



Five clusters of specimens

Diagram 10.9b. 5 clusters of spore dimensions (35 samples)



Five clusters of specimens

Diagram 10.9c. 5 clusters of spore dimensions (35 samples)

Table 10.15 Basidia measurements in two groups (23 samples)

Melbourne Unknown *Psilocybe* Species	**Basidia 1**	**Basidia 2**
2103829	24.6	6
2103697	22.7	14.3
2054573	28.6	9.6
2025255	27.7	11
2362243	25.9	8.55
2322085	26.5	8.9
2112647	32.1	8.65
2367138	24.6	7.05
2367327	31.7	10.6
2367329	20.8	8.7
2358338	23.9	7.8
2363659	19.5	8.3
2358340	26.4	7.8
2321893	21.9	6
269073	29.9	11.8
269071	32.5	12.5
228403	32.7	9.2
269070	26	8
2341406	25	9
2317471	20.3	7.7
2320554	21.1	7
2335174	24	7
2321029	28.6	11.3

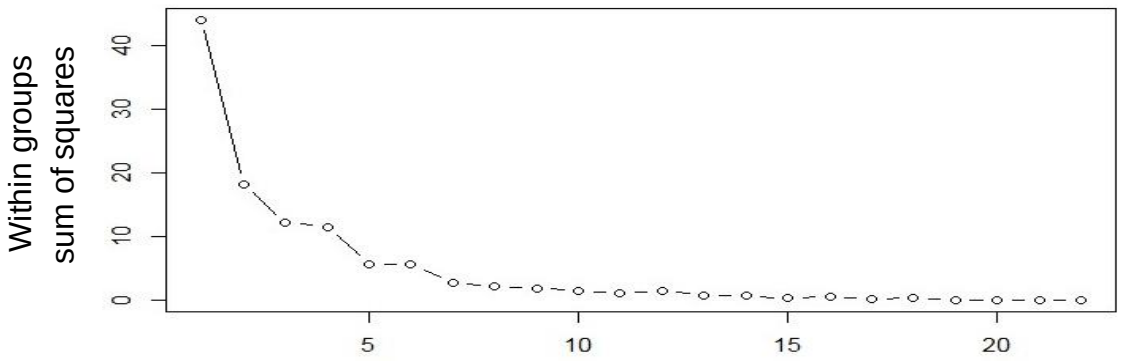


Diagram 10.10 Clustering based on dimensions of basidia characteristic (5 clusters)

Table 10.16 Basidia clustering (measurements of dimensions) (23 samples)

Melbourne Unknown *Psilocybe* Species	**cluster**
2103829	1
2367138	1
2321893	1
2335174	1
2103697	2
2025255	2
269073	2
269071	2
2321029	2
2362243	3
2322085	3
2358338	3
2358340	3
269070	3
2341406	3
2367329	4
2363659	4
2317471	4
2320554	4
2054573	5
2112647	5
2367327	5
228403	5

Diagram 10.11 (a-b-c) 5 Clusters of basidia dimensions for 23 samples

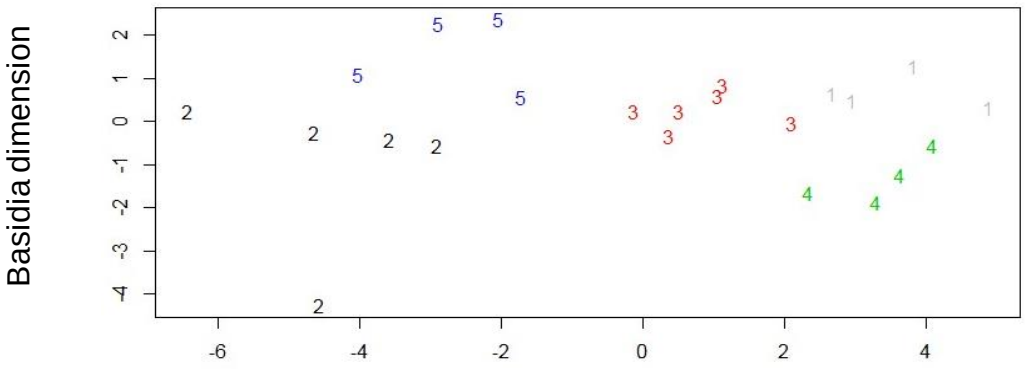


Diagram 10.11a. 5 Clusters of basidia dimensions for 23 samples

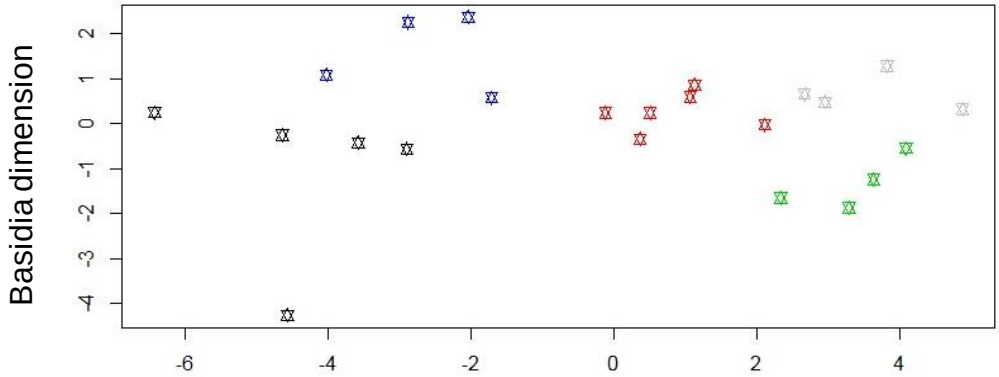


Diagram 10.11b. 5 clusters of basidia dimensions for 23 samples

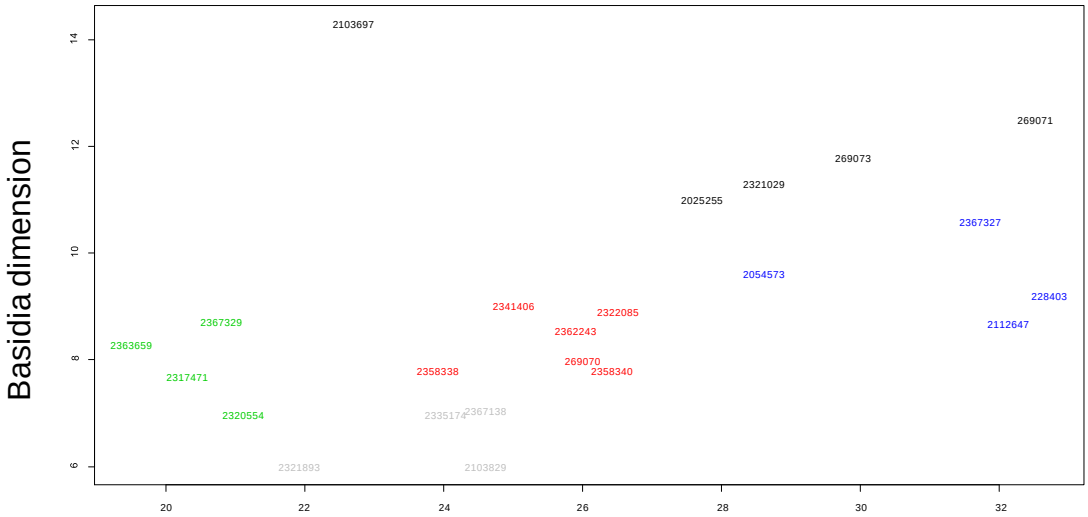


Diagram 10.11c. 5 clusters of basidia dimensions for 23 samples

Table 10.17 Clustering of cheilocystidia dimensions (19 samples)

Cheilocystidia		
Melbourne Unknown *Psilocybe* Species	**(Mean Length × Mean Width) µm**	**cluster**
2103697	30 × 14	2
2054573	25 × 7.50	6
2025255	29.25 × 11.25	2
2322085	29.40× 12	2
2112647	31 × 8.50	5
2367138	31.60 × 6.20	3
2367327	47.50 × 10.50	4
2367329	28.50 × 6	6
2358338	37.60 × 5.60	3
2363659	29 × 6	6
269073	30 × 7	6
269070	27.50 × 6.75	6
2104350	35.60 × 7.60	3
2231581	22 × 5	1
2103668	19 × 7	6
2317471	26.70 × 6	6
2335174	24 × 3.50	1
2321029	60 × 10	4
2031497	33 × 9.60	5

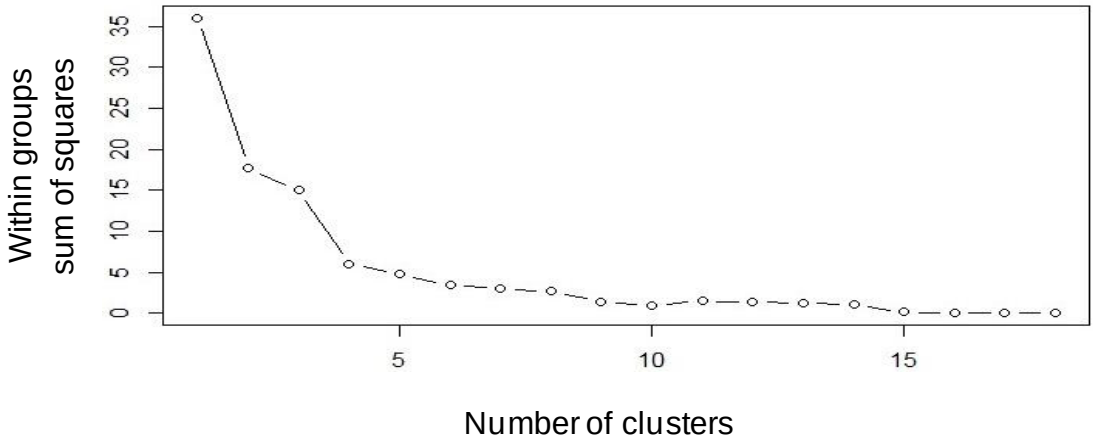


Diagram 10.12 Clustering based on dimension of cheilocystidia characteristics (6 clusters)

Diagrams 10.13 (a-b) 6 clusters of cheilocystidia dimensions (19 samples)

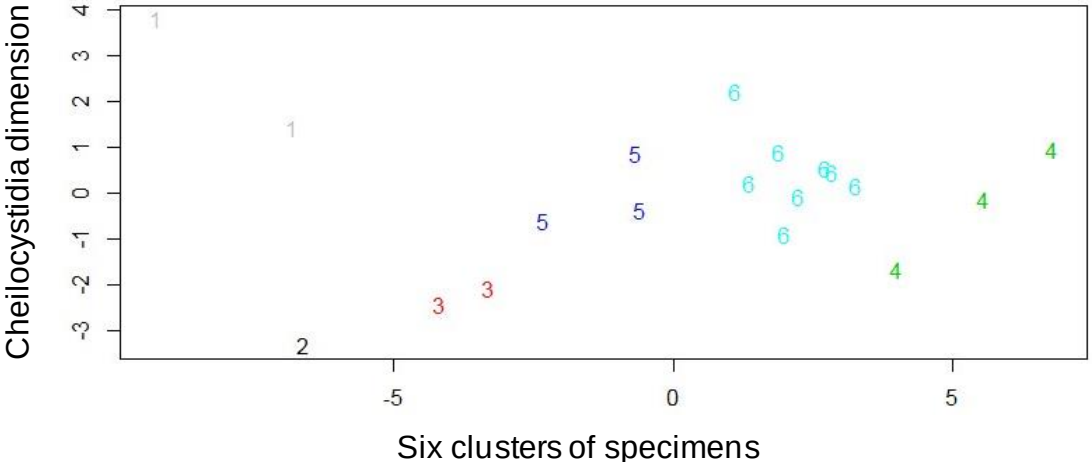


Diagram 10.13a. 6 clusters of cheilocystidia dimensions (19 samples)

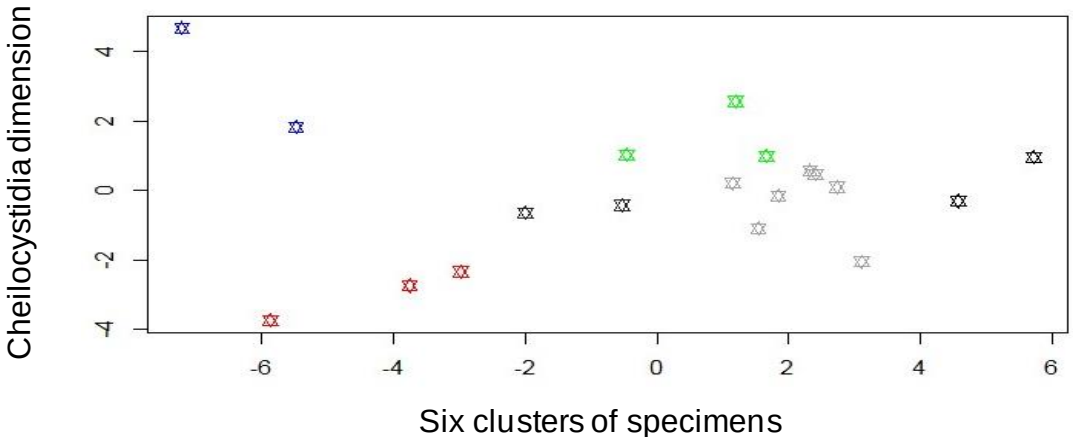


Diagram 10.13b. 6 clusters of cheilocystidia dimensions (19 samples)

Table 10.18 Spores and basidia dimension measurements clustering together

Melbourne Unknown *Psilocybe* Species	spores			Basidia	
id	s1	s2	s3	b1	b2
2103829	8.2	4.9	1.68	24.6	6
2103697	16	9.6	1.68	22.7	14.3
2054573	7.3	3.9	1.89	28.6	9.6
2025255	13.25	9.1	1.46	27.7	11
2362243	6.95	4.2	1.66	25.9	8.55
2322085	11.4	6.7	1.71	26.5	8.9
2112647	11.23	6.35	1.78	32.1	8.65
2367138	8.15	5.15	1.61	24.6	7.05
2367327	12.43	7.95	1.58	31.7	10.6
2367329	9.05	5.48	1.65	20.8	8.7
2358338	8	4.4	1.84	23.9	7.8
2363659	9.95	6.2	1.61	19.5	8.3
2321893	7.15	4.1	1.75	21.9	6
269073	13.35	7.6	1.76	29.9	11.8
269071	13.2	8.45	1.55	32.5	12.5
228403	11.55	7.65	1.51	32.7	9.2
269070	8.03	5.43	1.48	26	8
2341406	12.3	7.35	1.68	25	9
2317471	9.55	5.68	1.69	20.3	7.7
2320554	8.05	5.25	1.54	21.1	7
2335174	8.3	4.95	1.71	24	7

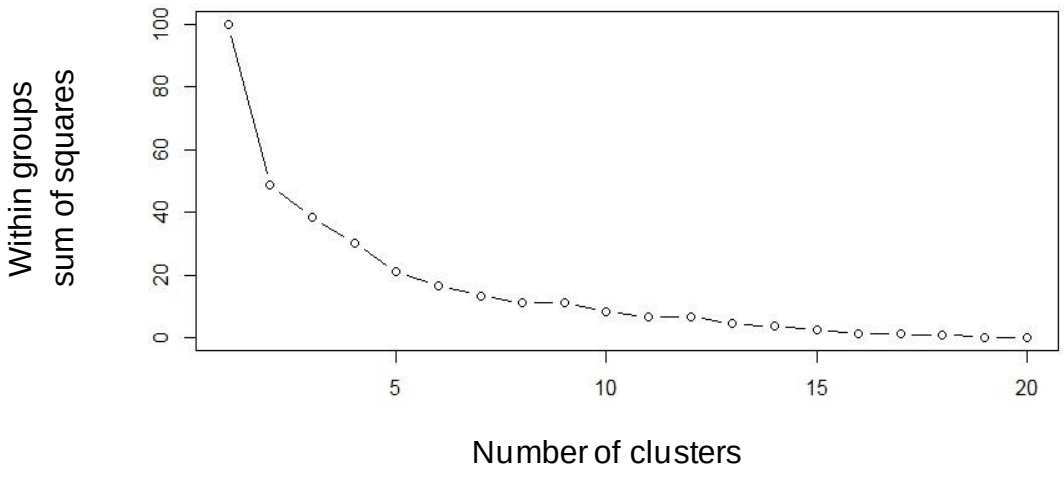


Diagram 10.14 Clustering based on measurements of dimensions of spores and basidia characteristics (7 clusters)

Table 10.19 Spore clustering (21 samples)

Melbourne Unknown *Psilocybe* Species	**cluster**
2322085	1
2112647	1
269073	1
2341406	1
2025255	2
2367327	2
269071	2
228403	2
2103697	3
2054573	4
2358338	4
2367329	5
2363659	5
2317471	5
2103829	6
2362243	6
2321893	6
2335174	6
2367138	7
269070	7
2320554	7

Diagrams 10.15 (a-b-c) Spore dimension measurements clustering (21 samples)

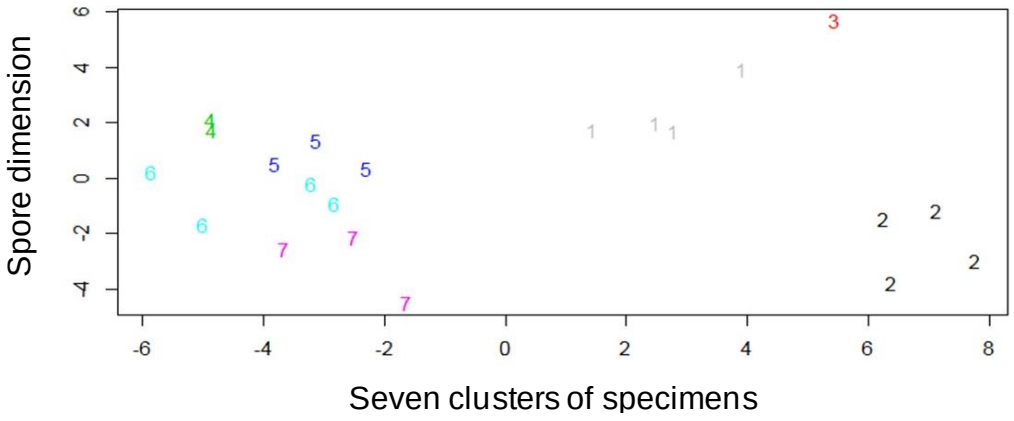


Diagram 10.15a. Spore dimension measurements clustering (21 samples)

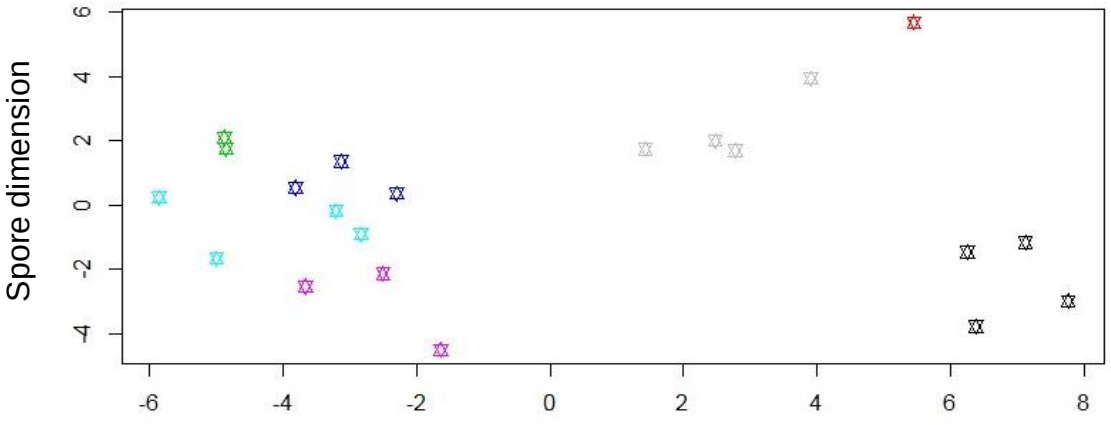


Diagram 10.15b. Spore dimension measurements clustering (21 samples)

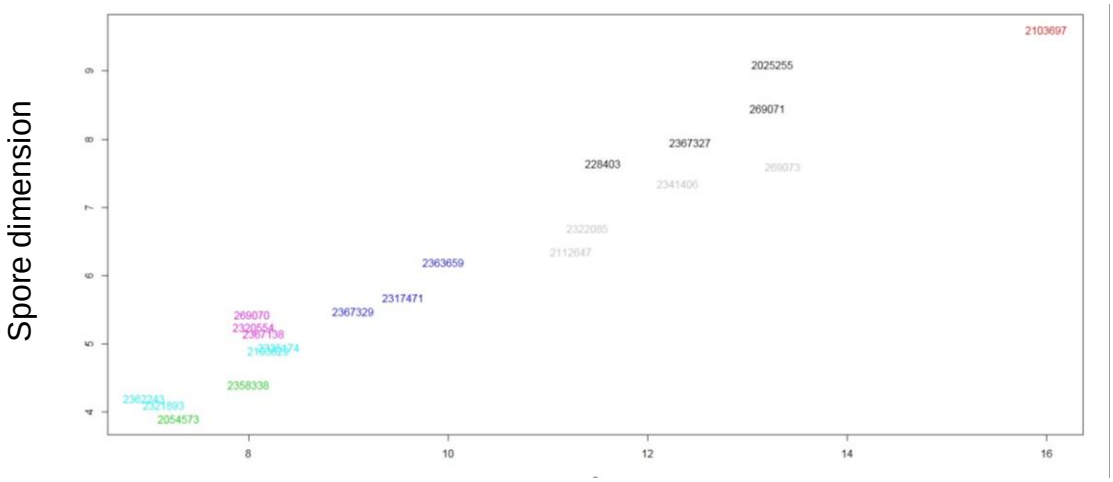


Diagram 10.15c. Spore dimension measurements clustering (21 samples)

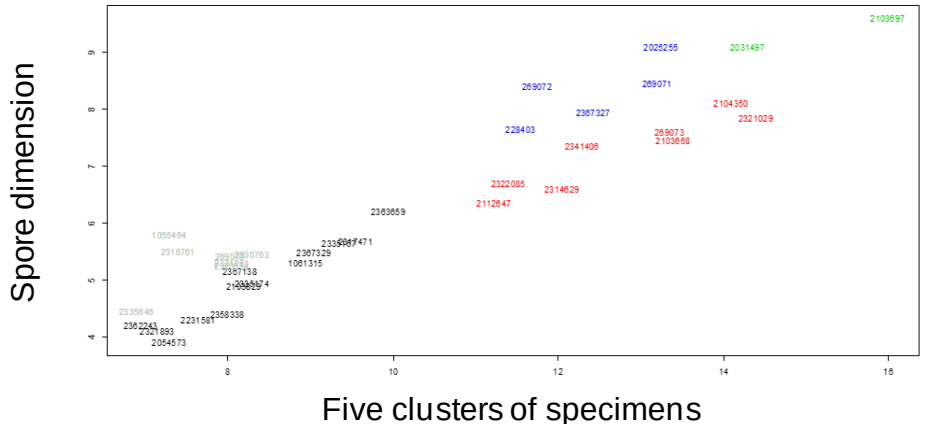


Diagram 10.16 Spore dimension measurements clustering (35 samples)

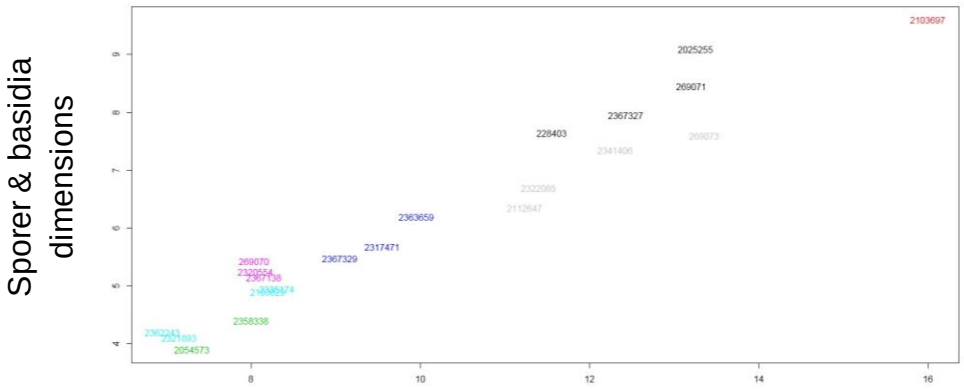


Diagram 10.17 Clustering of spore & basidia dimension measurements (main characteristics) (21 Samples)

Statistical Information;

 Packages R 3.3.2

1. Nb Clust
2. Fpc

 Codes

```
Mydata <- read.csv (file="mf.csv", head=TRUE, Sep=";") # read input data
na.omit (mydata) #omiting missed data data2=scale (mydata) #normalyzing data
Wss <- (nrow (data2)-1) *sum (apply (data2, 2, var))
For (i in 2: nrow (data2)-1) {wss[i] <- sum (kmeans (data2, centers=i) $withinss)}
Plot (1: length (wss), wss, type="b", xlab="Number of Clusters", ylab="Within groups sum of squares")
d=kmeans (data2, k) # k is the number of desired clusters
D$cluster
Plotcluster (data2, d$cluster, pch=11)
```

Figures 10.2 (a-b-c-d-e-f-g-h-i-j) Electronic microscope pictures; Spores/Basidia/ Cheilocystidia (Dimension µm; length and width)

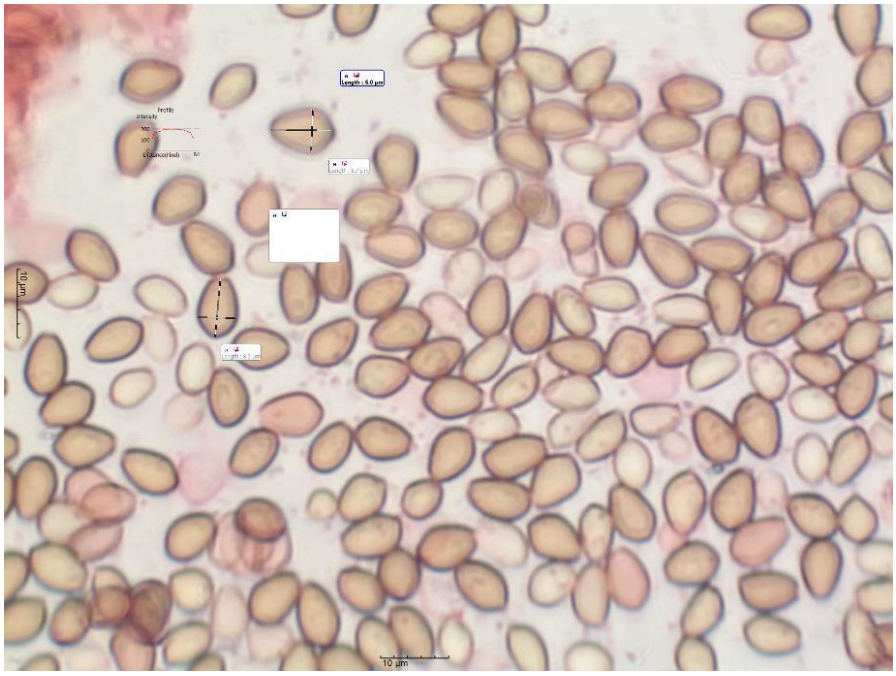


Figure 10.2a. Spores dimension measurements

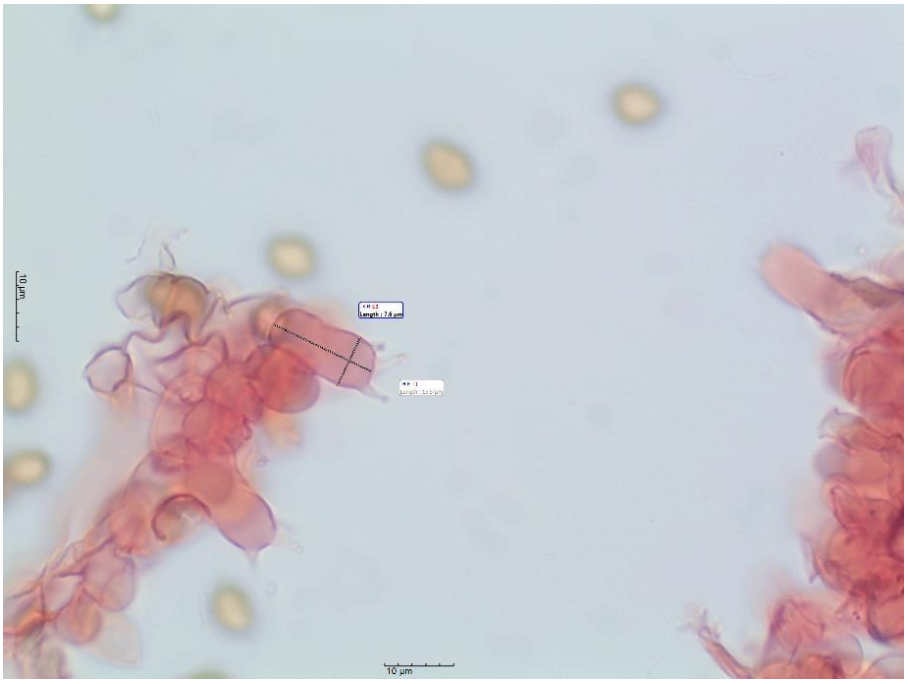


Figure 10.2b. Basidia dimension measurements

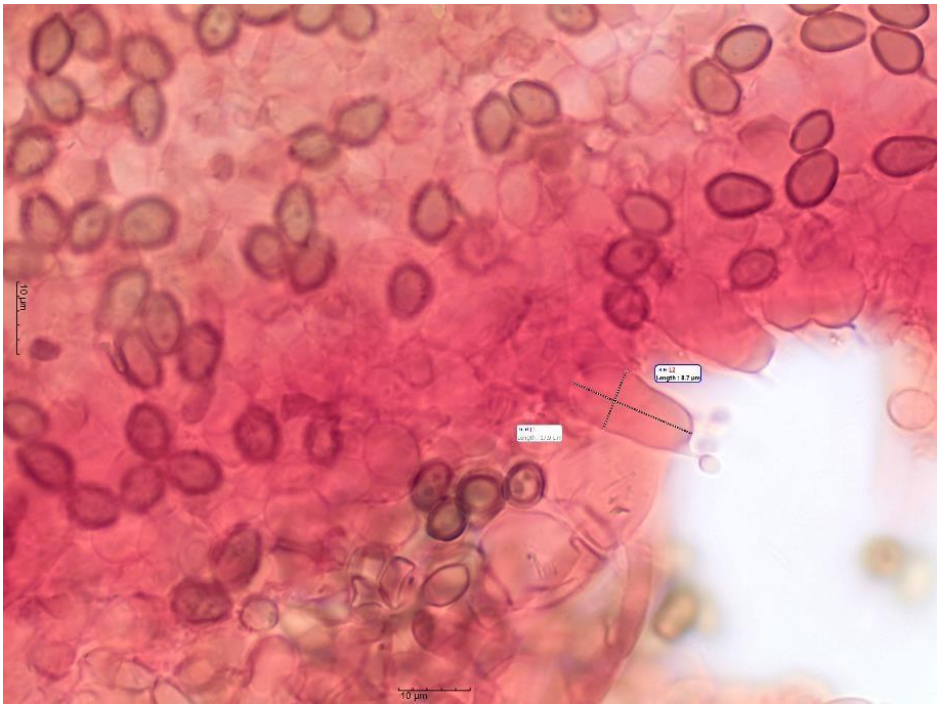


Figure 10.2c. Basidia dimension measurements

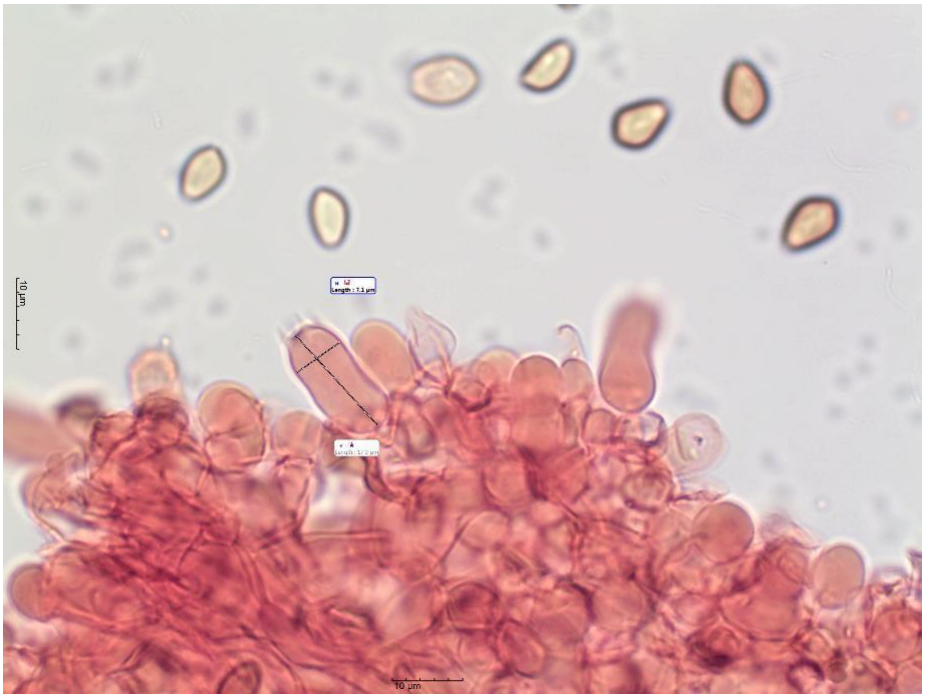


Figure 10.2d. Basidia dimension measurements

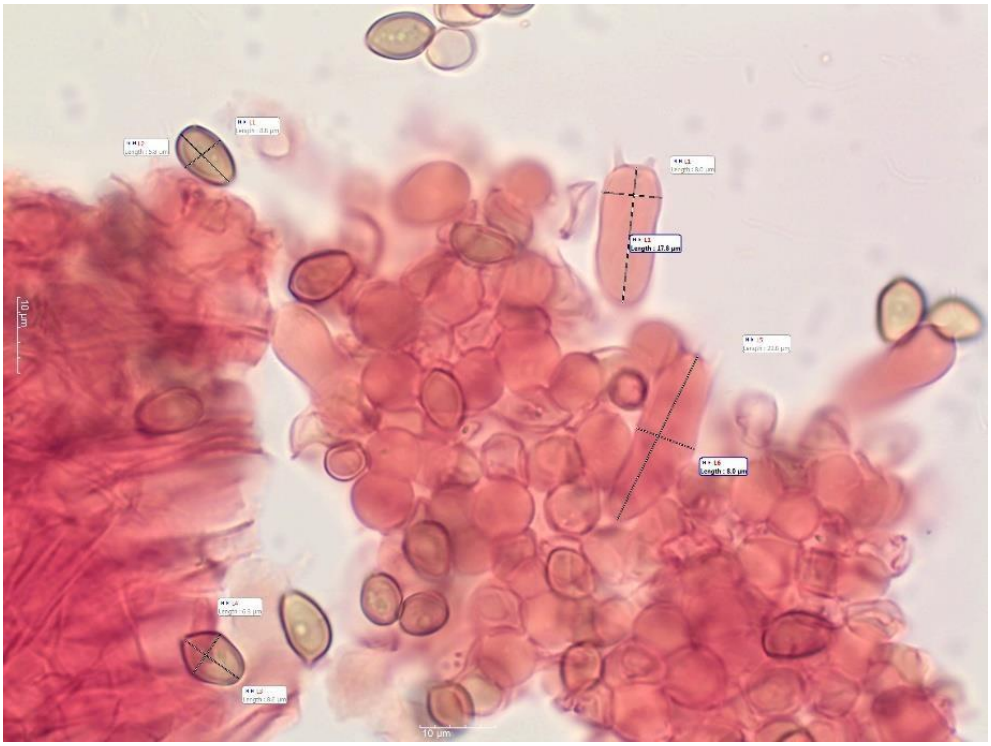


Figure 10.2e. Spores & Basidia dimension measurements

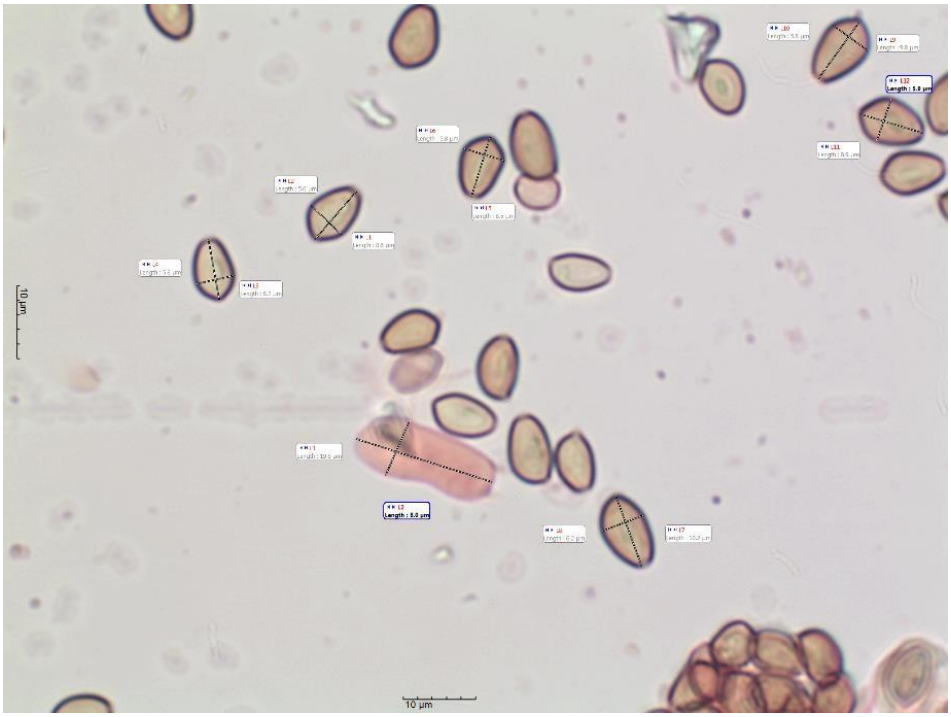


Figure 10.2f. Spores & Basidia dimension measurements

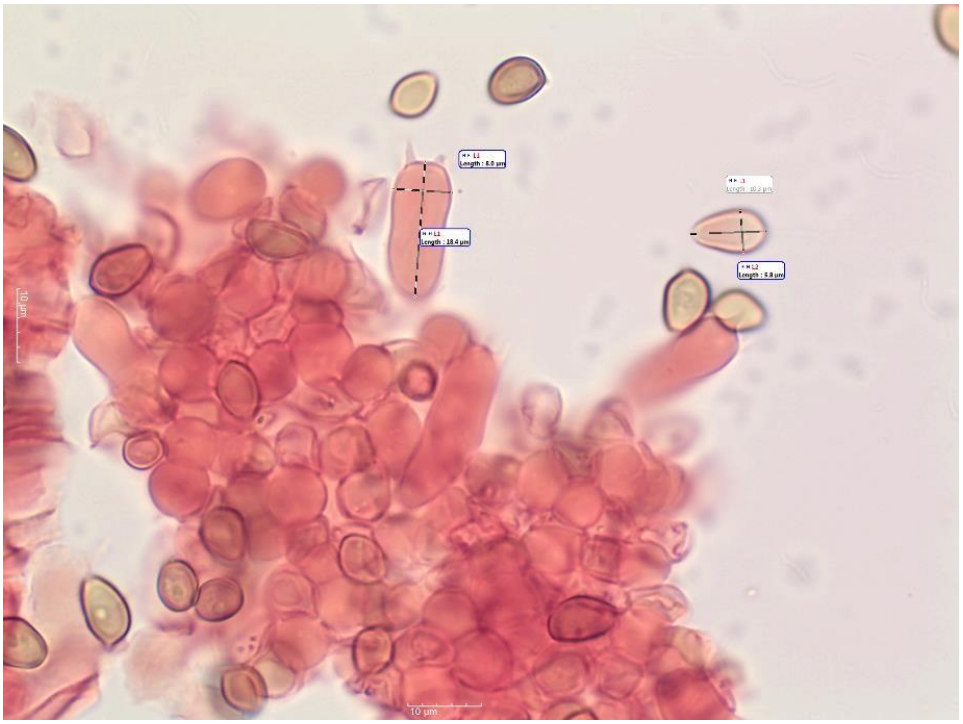


Figure 10.2g. Spores & Basidia dimension measurements

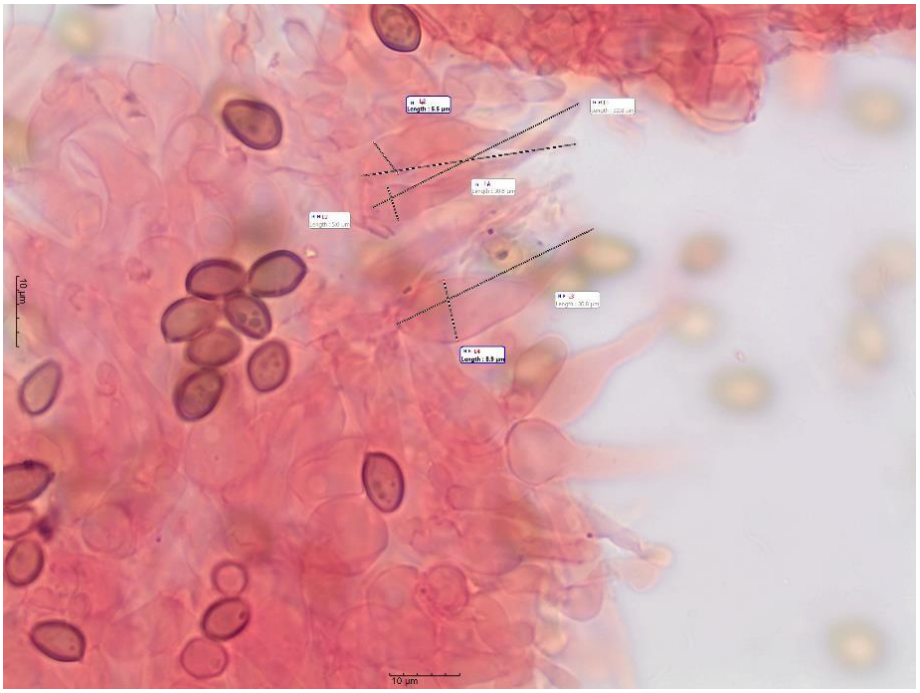


Figure 10.2h. Cheilocystidia dimension measurements

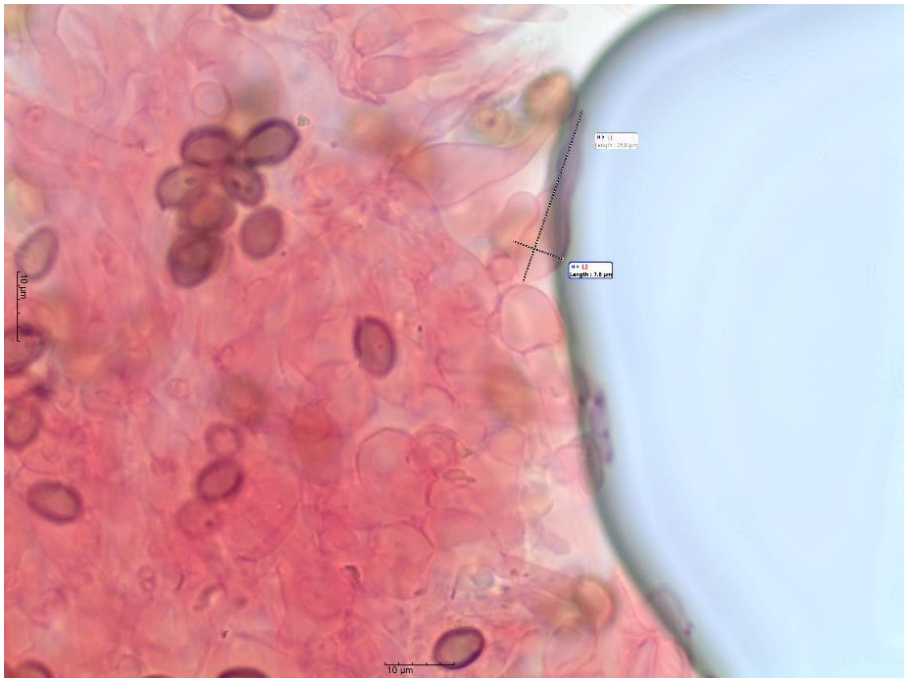


Figure 10.2i. Cheilocystidia dimension measurements

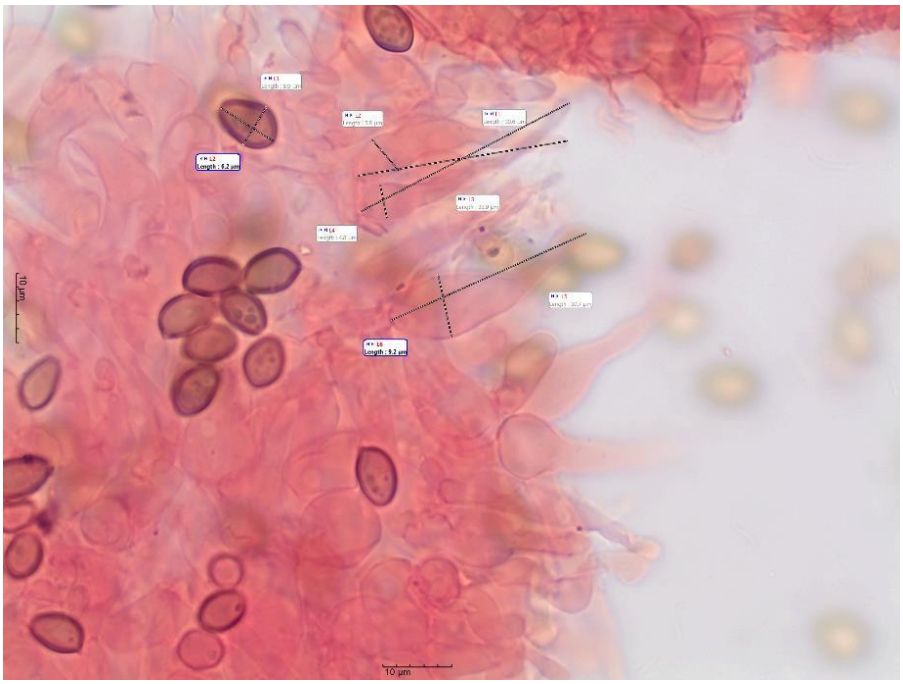


Figure 10.2j. Cheilocystidia dimension measurements

10.4 Fungal DNA Extraction (Isolation of Genomic DNA)

Based on a pilot study on various DNA extraction techniques, total genomic DNA is extracted from the fungal tissue, following the protocol described by the magnetic method, as showed the maximum efficient. The process followed the procedures, delivered by Qiagen (Kowalczyk, M et al. 2015).

Protocol: Purification of DNA from fungal tissues:

For fresh samples, first slightly brush off soil and dry them at 70°C, then mill them with laboratory mill. The milled mushrooms are put in labelled air-tight flasks and stored at 4 °C (Wandati, Kenji & Onguso 2013).

Procedure; 1) place 2 ml microcentrifuge tubes containing 1 stainless steel bead (5mm mean diameter) into the insert of the Tissue-Lyser LT Adapter (For loose pippte tubes always autoclave them), 2) Determine the amount of fresh plant-fungal material. Do not use more than 100 mg per sample, 3) Transfer the weighed tissue to the precooled tube and incubate for another 30 min on dry ice (Dry Ice temperature: - 78.5 °C) or -80 freezer. Alternatively, fungal tissues can be flesh-frozen in liquid nitrogen (Liquid Nitrogen temperature: -196 °C) and macerated prior to transfer to the precooled tubes. In this case, the additional 30 min incubation on dry ice is not necessary, 4) Place the precooled insert with sample tubes into the base of the Tissue-Lyser LT Adapter, which is attached to the Tissue-Lyser LT. Place the lid on the Tissue-Lyser LT Adapter over the insert, and screw the knob until the lid is securely fastened, 5) Immediately operate the Tissue-Lyser LT for 2 min at 50 Hz, 6) Remove the sample tubes from the insert of the Tissue-Lyser LT adapter, and incubate at room temperature (15-25°C) for 1 min to avoid freezing lysis buffer in step 7, 7) Add the appropriate volume of lysis buffer (e.g., Buffer AP1) to each tube, and proceed with DNA purification.

Note; Put fungi tissue straightaway into the liquid nitrogen for a few minutes for the first step instead of stage 1 / 2 and 3 (Větrovský et al. 2016).

Nuclear DNA can be extracted from a small piece (8–64 cubic mm or 10-50 mg) of dried gill, pileus, lamellae, basidiome and sporocarp tissues (Osmundson et al. 2013) and stored in DMSO (Dimethyl sulfoxide) buffer overnight or from frozen

fungal biomass (Hosaka, K & Uno 2013) using an adapted (CTAB) cetyltrimethylammonium bromide 2% (2 g) method with liquid nitrogen (Matheny et al. 2002). Genomic DNA is isolated from 25 mg dried herbarium specimens with the DNeasy. The enzyme for DNA extraction is DNA polymerase. The DNA quality and quantity is verified using a nanodrop spectrophotometer. Concentration and size profiles are determined on a Bioanalyzer using a High Sensitivity DNA chip (Staats et al. 2013). The ideal DNA concentration is: 0.3 ng to 1 ng (INYOD et al. 2017). To determine the quality of purified DNA, the DNA is run on 1% gel electrophoresis. For fungi, entire genomic DNA is extracted from 30–64 mg of dried herbarium material. The quantity is measured using a Nano-Drop 1000 spectrophotometer. Researchers currently make use of several different approaches for extracting DNA from fungal herbarium specimens ('Fungal gDNA Mini Kit') (Xcelris Genomics, Ahmedabad, India). 50 ng of DNA are used for species identification.

DNA in specimens degrades with time. This process is inevitable, because DNA molecules degrade as a consequence of hydrolysis, alkylation and oxidation even under a perfect circumstance, i.e., continuous low temperature. It is also claimed that the quality of DNA depends on how specimens are collected, prepared and retained temperature and time for drying specimens are two critical aspects for preserving quality DNA. Low DNA quality cannot be sequenced. Although drying by low heat (42°C or less) is often preferred to preserve high quality DNA. The lysis of cells to release DNA in the external environment is the most critical stage. We have reported, by comparing the DNA quality from phylogenetically distantly related mushroom species, that drying temperature does not significantly affect the DNA quality for subsequent studies (Hosaka, K & Uno 2013).

Whole DNA yields extracted from herbarium specimens ranged between 2400 and 45000 ng. The DNA is classically extremely degraded with DNA fragment sizes generally less than 1kb. Significantly, the quantity and quality of sequence data generated from historical specimens is not inferior to sequence data of fresh materials of the same species, displaying that high quality NGS libraries can be generated from Nano-gram quantities of historical DNA. Alternative long DNA fragments are simply hardly recovered from ancient specimens. Since there are

complications amplifying the regions longer than 600 bp from old (10 year or older) specimens stored in the herbarium, we assume that DNA in such specimens is vastly degraded due to the ages and fumigation (Bakker 2018). Five factors are used to assess the quality of DNA namely: (i) DNA condition,

(ii) colour, (iii) spectral absorbance ratio (A260/280), (iv) Final concentration (ng DNA/µL purified DNA) and (v) Purified PCR amplification products of ITS and nLSU. The DNA condition is categorized as (1) high molecular weight DNA, no degradation, (2) rather degraded, but still showing a band with high molecular weight DNA, or (3) extremely degraded and / or with a low molecular weight DNA band.

A positive control of known mushroom DNA is used, and negative control contained all reagents except DNA template is done to check the existence of contamination (Hussein et al. 2014). Standard precautions to decrease contamination are employed through, such as using dedicated pipettes with filter tips; bleaching of forceps/pestles; and sample accessioning, DNA extractions and treating of samples for Illumina sequencing are performed in distinct laboratories.

When we deal with the type specimens, some problems include the ages of specimens and poor quality of DNA are showed. While the ages of specimens and degrees of degradation in DNA, are not severely correlated, thus to save high quality DNA in type specimens, DNA from historically important specimens should be extracted as soon as possible or small pieces of specimens should be deposited under more ideal conditions for future DNA studies. More vitally, many herbarium specimens have been fumigated by a variety of chemicals to prevent insect and fungal damage. Many widely accessible fumigants are known to highly efficiently degrade DNA, among which the mixture of methyl bromide and ethylene oxide is confirmed to be one of the poorest in terms of DNA degradation. Perfectly, all specimens should be treated by fumigants, not affecting DNA, such as sulphuryl fluorid or not fumigated at all, but many traditionally important specimens, especially the type specimens, have already been hurt from such fumigants for a long time. One way of stopping further degradation of DNA, is a cryopreservation in liquid nitrogen or ultra-deep freezer

at - 80°C. If such services are not available, use of some organic solvents, such as acetone, or DNA preservation buffers is advised (Hosaka, K & Uno 2013).

For decades, a common practice for field preparation, especially in the tropics, was alcohol drying, also known as the Schweinfurt method, to prevent specimens from mould damage. Unfortunately, use of alcohol drying as a temporary fixative is known to have destructive effects on DNA. In general, fragments shorter than 300 bp can now be extracted from a broad range of historical specimens. DNA can be notoriously difficult to extract from fungal herbarium tissues extraction. In addition, historic sample preparation methods can significantly affect DNA recovery success. There is always a probability that fungi, that do not release their DNA, will not contribute to diversity or that vigorous extraction procedures can outcome in extremely fragmented DNA, generating chimeric PCR products (Jeewon & Hyde 2007).

Many researchers have been discouraged from using historical specimens in molecular studies because of both generally limited success of DNA extraction and the challenges associated with PCR-amplifying highly degraded DNA. In today's next-generation sequencing (NGS) world, opportunities and prospects for historical DNA have changed dramatically, as most NGS methods are actually designed for taking short fragmented DNA molecules as templates. Using a standard multiplex and paired-end Illumina sequencing approach, genome-scale sequence data can be generated reliably from dry-preserved plant, fungal and insect specimens collected up to 115 years ago, and with minimal destructive sampling.

Furthermore, NGS of historical DNA enables recovering crucial genetic information from old type specimens that to date have remained mostly unutilized and, thus, opens up a new frontier for taxonomic research as well. In today's next-generation sequencing (NGS) world, prospects for historical DNA have changed intensely, as most NGS approaches do not trust on large, integral DNA templates but are truly considered for taking short fragmented molecules (100–400 bp) as templates. DNA isolated from historical specimens provides exactly that, the process of specimen preparation which might include exposure to heat (plants / fungi) or killing using ethyl acetate or formalin (insects), is known to

cause significant genome fragmentation by incidence of extensive double-stranded breaks, and to be independent of specimen age. However, the application of NGS technologies to ancient DNA from paleontological and archaeological archives has been definitely established and its application to historical museum specimens is rare and so far restricted to mammals, snails and plants.

The most innovative and novel fungal extraction techniques;

1) DNA from specimen is extracted, using the modified cetyltrimethylammonium bromide (CTAB) extraction, following glass milk purification methods (as summarized by (Hosaka, T et al. 2009) (Hosaka, K & Castellano 2008). Briefly, samples are ground in liquid nitrogen using a mortar and pestle, incubate in CTAB buffer (2 % CTAB, 100-mM Tris pH 8.0, 20-mM EDTA, 1.4-M NaCl) at 65 °C for 1 h, and proteins are removed using the mixture of chloroform: isoamyl alcohol (24:1). The materials are further purified using 6-M sodium iodine buffer (1-M Tris pH 6.8, 2-M Na2SO3) with glass milk, wash with ethanol / buffer solution (10-mM Tris pH 7.4, 1-mM EDTA, 100-mM NaCl, 50 % EtOH), and finally eluted in 100 μl of Tris-EDTA buffer (TE, 10-mM Tris–HCl pH 8.0, 1-mM EDTA).

2) DNA is isolated from the gill tissues stored in DMSO buffer (Seutin, White & Boag 1991) (Hosaka, T et al. 2009) overnight. Tissues are first ground under liquid nitrogen, using a mortar and pestle. Ground tissues are then transferred to new 1.5 ml tubes using clean spatulas and CTAB buffer is added.

3) Conventional or optimized CTAB-based methods (Cubero et al. 1999) (Ristaino, Groves & Parra 2001) (Telle & Thines 2008) or increasingly, solution-based or column-based commercial kits (Crouch & Szabo 2011) (O'Gorman & Fuller 2008) (Sokolski, Piché & Bérubé 2004). Briefly, dried sporocarp samples are pulverized using a bead mill, suspended in a CTAB extraction buffer and subjected to 3 rounds of freeze-thaw consisting of alternating 3 min treatments in

dry ice and a 70°C heating block, followed by a 30 min incubation at 70°C. Samples are subsequently treated with phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged for 15 min at 13,0006g, then DNA is purified from the supernatant (using the Gene-Clean Turbo kit (QBiogene, Inc.).

4) DNA is extracted from dried fruit body specimens using a modified cetyltrimethylammonium ammonium bromide (CTAB) extraction method as detailed in (Muruke et al. 2002). This method has proven to be useful for DNA extraction of herbarium specimen, field collected specimen and woody basidiomata bearing high content of secondary metabolite and high polysaccharide content (Rocha et al. 2011). The mushroom samples that are previously collected and preserved in silica gel are crushed to powder using a mortar and pestle with liquid nitrogen added to aid the process. About 30-50 mg of powered mushroom samples are added to 1.5 mL Eppendorf tubes and labelled accordingly. To each sample 450 µL of preheated (65°C) extraction buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, CTAB [2.5% w/v], Dithiol threitol solution (DTT) [1% v/v] and polyvinyl pyrrolidone (PVP) 1% w/v) are added. The samples are incubated in a water bath at 65°C for 45 minutes. An equal volume (450 µL) of chloroform-isoamyl alcohol (24:1) are added to each sample and the tubes are inverted twice to mix. The mixtures are centrifuged at 12000 rpm

(Eppendorf centrifuge 5424) for 15 minutes. A fixed volume of 400 µL of supernatants is transferred to new 1.5 mL Eppendorf tubes. To precipitate DNA

0.7 volumes (280 µL) of cold isopropanol (stored at - 20 °C) is added to the samples and inverted twice to mix to aid precipitation. The samples are incubated at - 20 °C for three hours and then centrifuged at 12000 rpm (Eppendorf centrifuge 5424R) for 15 minutes at - 4°C. The supernatants are decanted and the pellets (crude DNA) are air dried for 30 minutes. Samples are washed twice with 70% ethanol and centrifuged at 12000 rpm (Eppendorf centrifuge 5424R) for 15 minutes at - 4°C and supernatants are decanted. The pellets are suspended in 200 µL of low-salt TE (10 mM Tris, 0.1 mM EDTA [pH 8]). Three (3) µL of RNAse (10 mg/mL) is added to each sample and followed by incubation in a

water bath at 37 °C for 30 minutes. After incubation, 450 µL of chloroform-isoamyl alcohol (24:1) are added to each sample and the tubes inverted twice to mix. The samples are centrifuged at 12000 rpm (Eppendorf centrifuge 5424R) for 15 minutes at - 4°C. A fixed volume of 150 µL of the supernatant layer is transferred to the fresh-labelled Eppendorf tubes 1.5 mL.

Purification is done by adding 315 µL of ethanol-acetate solution (30mL EtOH, 1.5mL 3 M NaOAc [pH 5.2]) to each sample and kept in – 20°C for three hours. Samples are centrifuged at 12000 rpm (Eppendorf centrifuge 5424R) for 15 minutes at - 4°C. The supernatants are decanted and the pellets are washed with 100 µL of 70% ethanol. The samples are centrifuged at 12000 rpm (Eppendorf centrifuge 5424R) for 15 minutes at - 4°C, the supernatants are decanted, and pellets are air-dried for 30 minutes. They are finally suspended in

100 µL of low-salt TE (10 mM Tris, 0.1 mM EDTA).

5) Genomic DNA is isolated from 75 mg of lyophilized mycelium, using the following modified CTAB extraction procedure. Lyophilized tissue is pulverized with glass beads in a FastPrep1 instrument (Bio101, Carlsbad, CA) for 5 s at 4000 rpm. Pulverized tissue is incubated in 500 ml CTAB on dry ice for 2 min, and then thawed at 75 x for 2 min. DNA is purified in phenol:chloroform:isoamyl alcohol (25:24:1), further cleaned (by using the Geneclean1 Turbo Nucleic Acid

Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions) and eluted in 30 ml ultra-pure water.

6) DNA extraction from both dried fruiting bodies and fresh samples in CTAB buffer (preserved for over 6 months) and mycelia is done according to (Henrion, Chevalier & Martin 1994) with some modifications by (Danell 1994). About 20 to 50 mg sample of dry or fresh (preserved in CTAB buffer) fruiting bodies or mycelia are directly crushed using plastic pestle in 1.5 ml Eppendorf tubes containing 750 µl extraction buffer (5 M NaCl, 1 M Tris-HCL pH 9.0, 0.5 M EDTA, 10% CTAB, 0.2% -Mercaptoethanol) until the mixture is completely

homogenized. The tubes are then incubated at 65°C for 60-90 minutes. After centrifugation for 5 minutes at a maximum speed (14,000 rpm in a microcentrifuge), an equal volume of chloroform is added to the supernatant and vortexed for 10 seconds followed by 10 minutes centrifugation at maximum speed. To precipitate DNA, 2 volumes of pre-chilled iso-propanol is added to the supernatant mixed gently and incubated at -20°C for 2 hours. The DNA is pelleted by centrifugation for 20 minutes at maximum speed and washed twice with 300µl of ice cold 70% ethanol. After drying for 1 hour at 60°C (or overnight at room temperature), the DNA pellet is dissolved in 50 µl of ET buffer (10 mM Tris-HCL pH 8.0 and 1 mM EDTA) and stored at 4°C until use.

7) DNA is extracted from specimens as following. Briefly, dried sporocarp samples are pulverized using a bead mill, suspended in a CTAB extraction buffer, and subjected to 3 rounds of freeze-thaw consisting of alternating 3 min treatments in dry ice and a 70°C heating block, followed by a 30 min incubation at 70°C. Samples are subsequently treated with phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged for 15 min at 13,0006g, then DNA is purified from the supernatant (using the Gene-Clean Turbo kit (QBiogene, Inc.).

8) DNA Isolation: (1-20 mg of total genomic DNA is needed) isolate gills or a piece of the cap, OR a single root-tip or ECM morphotype, using sterile forceps and/or surgical scissors. Place tissues in 1.5 ml tubes that fit the micro-pestles (tubes from SSI-Plastics, ref: 1210-00); (Best practice; always load from lowest concentration to highest concentration).

a. Steps c to l must be performed under the fume hood. Wear gloves and lab-coat.

Read MSDS files of β-mercaptoethanol and Phenol: Chloroform: Isoamyl.

These two solutions are toxic.

b. Warm up the Carlson lysis buffer to dissolve CTAB that precipitates at 4°C.

c. Prepare the volume of lysis buffer according to the amount of samples to extract: 400 µl of Carlson lysis buffer per sample + 60 µg of RNAse A (0.6 µl of RNAse A at 100 mg.ml) per sample + 0.8 µl of β-mercaptoethanol per sample.

d. Start the heat block and / or a water bath, the temperature is set to 65°C (Shake samples in water bath every 10 min with hands).

e. Fill two beakers with liquid nitrogen; one is for freezing your pestles, the other one for soaking the bottom of the 1.5 ml tubes containing the pieces of fungal tissue.

f. Crush by hand all your samples previously frozen in liquid nitrogen. Leave the pestle in the tube.

g. Add 401.4 µl of lysis buffer in each tube, incubate them 10 min at 65°C and crush again your samples with the pestles. At this step you can use the fancy Pestle Mixer or do it by hand. Remove the pestles and leave them soaking in a beaker with tap water.

h. Incubate your samples at least 1 hour at 65°C. Gently mix them from time to time (do NOT vortex).

i. Meanwhile, clean the pestles and fill 1.7 ml tubes with 70 µl of ammonium acetate (7.5M) and 600 µl of cold isopropanol. Store these tubes at -20°C or 10 min at - 80°C. At this step the new tubes can be labelled with the laminated tape printed out with the labeller.

j. Add 400 µl of Phenol: Chloroform: Isoamyl (25:24:1), shake the tubes (but do NOT vortex). Do it under the fume hood, take care so no small drops escape from the tubes.

k. Centrifuge 15 min, max speed. As much as possible, do the transfer from centrifuge under the fume hood to limit phenol-chloroform smell spreading in the lab.

l. Transfer supernatants in new 1.7 ml tubes containing 70 µl of ammonium acetate (7.5M) and 600 µl of cold isopropanol.

m. Mix gently by inversion for 30-60 s and incubate tubes at least 1h (could be overnight) at -20°C. Cool down the centrifuge to 4°C, 10 min before the end of tube incubation at -20°C.

n. Centrifuge 15 min, max speed at 4°C.

o. Remove isopropanol (take care of the pellets) with a vacuum pump (or by pipetting but it is less efficient and more cumbersome) and wash the pellet with 800 µl of cold 70% ethanol.

p. Remove ethanol with a vacuum pump and dry pellets 30 min at 50°C on the thermo block. Warm up TE-8 buffer at 50°C.

q. Dissolve pellets in 50 µl of TE-8. Incubate tubes 20-30 min at room temperature, vortex and centrifuge quick spin. If pellets are still not dissolved, store overnight the tubes in the fridge. Store then at - 20°C.

r. It is usually necessary to dissolve 1/10 gDNA before PCR. For pallets use chilled ethanol.

At the end of the extraction, do not forget to clean the centrifuges to remove water and phenol-chloroform traces, clean as well the racks you used.

9) Micro pestle Plant DNA extraction under liquid nitrogen.

Centrifugation Protocol: Use the PALL glass fibre filtration 1.0 µM 350-µL well plates (no. 5031) for Centrifugation;

a. Add a small amount of sample (~1- 2 mm^3) to each well of a 96-well PCR plate (for example the Sorenson 96-well ultra-Plate; no. 21970). Instruments should be sterilized between samples to avoid cross contamination. Last well can be left blank and used as a negative control.

b. Mix 5 mL of Lysis Buffer (LB) and 0.5 mL of Proteinase K (20 mg/mL) in a sterile container and dispense 100µl to each well. Cover each row with caps and incubate at 56°C overnight (8 to 16 hours) to allow digestion.

c. Centrifuge at 1000 rcf for 1minute.

d. Add 100ul of Binding Mix (BM) to each sample. Mix by pipetting up a few times.

e. Remove cap strips/cover and transfer the lysate (about 150µl) from the wells of microplate into the wells of the PALL glass fibre filtration (GF) plate placed on top of a square-well block. Seal the plate with adhesive cover.

f. Centrifuge at 1500 rcf for 10 min to bind DNA to the GF membrane.

g. Add 250µl of Protein Wash Buffer (PWB) to each well of the GF plate. Seal with a new adhesive cover and centrifuge at 1500 rcf for 5 min. Discard the flow through.

h. Add 300µl of Wash Buffer (WB) to each well of the GF plate. Seal with a new cover and centrifuge at 1500rcf for 10 min.

i. To avoid incomplete WB removal, open the cover to relieve the vacuum that have formed in the wells, seal the plate again and centrifuge the plates again at 1500rcf for 5 minutes. Discard the flow-through.

j. Repeat steps h and i.

k. Remove the cover. Place the GF plate on a clean square-well block and incubate at 56°C for 30 min to evaporate residual ethanol.

l. Position a PALL collar on a collection plate (Sorenson 96-well Ultra-Amp) and place plate and collar on top of a clean square-well block. Place GF PALL plate with DNA bound to the membrane on top of a PCR plate. Dispense 50µl of 0.1x TE buffer or water, pre-warmed at 56°C, directly onto the membrane of each well of GF plate and incubate at room temperature for few minutes and then seal plate.

m. Centrifuge at 1500rcf for 10 minutes to collect the eluted DNA. Remove the GF plate and discard it.

n. Cover DNA plate with aluminium foil. Keep at 4°C for temporary storage or at 20°C for long-term storage.

Micro pestle Plant DNA extraction under liquid nitrogen; Requirements:

- Small Mortar

- Metal tweezers
- 5ml test tube
- Micro pestle
- Liquid nitrogen
- Plant Material
- DNeasy mini kit

Instructions;

a. Firstly, fill the mortar with liquid nitrogen

b. Place the test tube and micro pestle in the mortar and allow them to freeze

c. Refill the mortar with liquid nitrogen as it evaporates

d. After about 5 min, remove the test tube with the tweezers

e. Fill the tube with roughly 1-2ml of liquid nitrogen from the mortar

f. Using the lid of the test tube, hang the tube over the lip of the mortar, leaving the tube in the liquid nitrogen in the mortar (This stops the liquid nitrogen from evaporating)

g. Collect a small amount of fungi tissue and place it in the test tube

h. Using the tweezers, collect the micro pestle and begin to grind up the sample in the tube

i. Continue to add liquid nitrogen to the mortar and it evaporates

j. Once the plant tissue is ground up into very fine particle, remove it from the mortar

k. Lightly place your finger over the opening of the tube to prevent any plant material escaping as the liquid nitrogen rapidly evaporates

l. Once the liquid nitrogen is gone, allow the tube to slightly thaw and add the lysis buffer and RNase from the DNeasy mini kit

m. Vortex the test tube

n. Transfer the mixture to a micro-tube and proceed with the DNeasy mini kit protocol

Stock solutions needed for DNA extraction: Twice distilled water

- Ethanol leaf extract
- Water-ethanol leaf extract
- Water methanol petroleum ether
- Ammonium acetate
- TE-8 1X
- TRIS 100x (1 M)
- EDTA 100x (0.1 M)
- Carlson lysis buffer

10.5 Next Gene Sequencing (NGS) / Genealogical Concordance (GC)

The conventional means of creating DNA sequence data to attain a barcode for a species or a specimen are through PCR amplification and Sanger sequencing of DNA barcode sequences from genomic DNA extracted from single specimens. For library assembly this is for well-identified specimens whereas for species discovery the specimen need not be defined. Sanger sequencing skill is capable of producing sequencing reads of up to 1000 bases and was the only tactic used for DNA sequencing for almost three decades but nextgeneration sequencing (NGS) devices are now beginning to direct the sequencing market. Next generation sequencing knowledge allows for the sequencing of millions of DNA fragments from thousands of DNA templates in comparable. The fungal DNA barcode, ITS has been revealed to be present as several variable replicas within an individual's genome, therefore leading to trouble in direct Sanger sequencing of amplicons. Competing signs are so weak that they do not impact the generation of solo barcode. Conversely approaches have also been advanced to independently tag amplicons (using a set of oligonucleotides with a known sequence) pool them into a single sequencing run and recover them bioinformatically. A decreased fault ratio and amplified sequencing throughput will more amplify the rate at which DNA barcodes can be formed with NGS technology (Shokralla et al. 2014).

In progress to encourage the practice of a uniform DNA barcoding approach containing of classifying a specimen based on a single worldwide marker i.e., the DNA barcode sequence. A perfect DNA barcode region or locus must have low intra-specific and high inter-specific variance (forming a barcode gap) and easy to amplify from most or all species in the target collection via widespread primers. Reference barcodes need to be derived from proficiently known vouchers deposited in biological assortments with online metadata and authenticated by obtainable online sequence chromatograms (Purty & Chatterjee 2016). The achievability of NGS for single specimen DNA barcoding for library preparation or specimen identification has been studied (Shokralla et al. 2014).

A DNA barcode in one or limited rather short gene sequences present in the genome which is irreplaceable enough to detect species. DNA barcoding is a suitable means for taxonomic arrangement and identification of species by sequencing a very short standardized DNA sequence in a well-defined gene. In this technique thorough information of the species can be achieved from a single case regardless to morphological or life phase characters. It is an operative technique in which extracted DNA from the collected sample is processed following the standard procedure. Identification of the species is carried out by amplifying extremely variable region like DNA barcode region of the nuclear chloroplast or mitochondrial genome with PCR. Region broadly used for DNA barcoding consist of nuclear DNA (ITS) chloroplast DNA and mitochondrial DNA. DNA barcode can be used as a tool for alignment unknown species, based on barcode sequence to earlier known species or new species. It likewise can be used as a supplement to other taxonomic datasets in the process of delineating species boundaries.

The set of DNA barcode markers have been applied to definite taxonomic sets of organisms and are providing priceless for understanding species boundaries community ecology practical trait evolution trophic interactions and the conservation of biodiversity. The application of NGS skill had extended the flexibility of DNA barcodes through the tree of life environments and geographies as new methodologies are discovered and developed. In order to characterize species Consortium for the Barcode of Life (CBOL) has nominated a small number of genes as supreme for DNA barcoding. Preferably one gene sequence

would be used to identify species in all of the taxa from viruses to plants and animals. On the other hand, ideal gene has not yet been found, so diverse barcode DNA sequences are used for animals, plants, microbes and viruses. DNA barcoding uses short gene sequences which, are well categorized portion of the genome. By coming on the great output sequencing technology such as NGS, the DNA barcoding has become more precise, fast and reliable in the last eras (Purty & Chatterjee 2016).

The barcoding protocol proposed here produces short barcode, 313 bp in length since they are more suitable for NGS and have an advanced amplification success rate than long barcodes. However, these barcodes deliver even less information than standard DNA barcodes that have been fairly criticized for being too short to be useful. Short barcodes are acknowledged to be less popular in assigning specimens to species and / or higher taxonomic ranks and species identification success rates for strictly linked species, are higher for studies using numerous genes (Meier et al. 2016). In Sanger sequencing full-length barcode fragments are involved as in NGS, shorter barcode fragments are involved. It is difficult to identify the species of the fungi based solely on morphological observation. Modern identification methods built on a genetic analysis are gaining popularity with some authors, considering genetic identification essential (Maruyama et al. 2003). A DNA-based identification technique will allow the identification of samples, when there is a lack of morphological features such as in the mycelium or spore stage (Nugent & Saville 2004).

Over the last few decades, there has been a paradigm shift in the methods, routinely used for the taxonomic identification of biological materials. Traditionally, identifications were achieved after rigorous examination of morphological characteristics and subsequent consultation with the appropriate authoritative taxonomic literature. However, in scenarios where the specimen is incomplete, traditional morphological methods can only generate reliable classifications at higher taxonomic levels. When morphology is added to molecular data in joined analysis, there is most often an increase in resolution compared to molecular data only. When molecular data is added to morphological data, the change in resolution is always positive. The result of

adding molecular data to morphological data is also statistically significant (Wortley & Scotland 2006).

Ordination likewise indicated that specimens from the same species are commonly dispersed, overlapping with specimens from other species. Even when the possibility of appropriately classifying a specimen to its species is high, the probability that members of other candidate species would wrongly be assigned to the same species, is also high (Bazzicalupo et al. 2017).

Molecular techniques have been hired, mainly including the application of hybridisation probes, PCR amplification of rDNA genes and other DNA fingerprinting methods. These include terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), amplified random intergeneric spacer analysis (ARISA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), oligonucleotide fingerprinting of rRNA genes or single-stranded conformation polymorphism (SSCP) and have been used often in combination with traditional techniques to analyse fungal community composition. Although rDNA has been the most broadly used gene for systematics studies, DGGE has been the most useful genetic fingerprinting performance to investigate complex microbial communities from a variety of environmental samples. Mainly this method involves separation of individual sequences (with different base composition and melting properties) from a mixture.

DGGE is the preferred environmental fingerprinting tactic as it: (1) allows large and multiple samples to be analysed simultaneously, (2) overcomes diversity bias from traditional attitudes (e.g. cultural methods), (3) can magnificently screen community shifts and succession over time, (4) permits the outlining of communities under diverse environmental situations (especially in degraded / polluted ecosystems), (5) makes it probable to obtain taxonomic evidence through phylogenetic analyses, and (6) gives a suggestion about the potential biological role of specific microorganisms in the sample (e.g. those that can be involved in the decay of organic material or degradation of pollutants) (Jeewon & Hyde 2007).

Most of the gene regions directed in community analyses are from the conserved 18S rDNA gene and are less than 600 base pairs, so that a reasonable DGGE resolution can be reached. This is, however, to the detriment of exact systematics and phylogeny. In many cases, the primer pairs used are specific to a group of fungi, whereas some at the same time can amplify DNA from absolutely unrelated organisms. Although DGGE is a promising tool, it can still underestimate fungal diversity. The number of bands depends on the resolution of the gels; this takes time to optimise and is hard to reproduce. The conventional method of mushroom identification is the use of macro- and micromorphological features. However, this method is not accurate and reliable due to phenotypic plasticity and intraspecific variability among the mushrooms, which could arise from mutations, or substrate and growth effects (Musa, Wuyep & Gbem).

One recent approach for establishing genetic relationships in medically essential fungi is by analysis of limitation fragment length polymorphisms (RFLPs) in their DNAs (Vilgalys & Hester 1990). Latest developments of molecular techniques have delivered a method of recognizing species on the basis of phylogenetic trees constructed from DNA data, i.e., phylogenetic species recognition (PSR). In many circumstances, PSR discovered phylogenetically different taxa that had not formerly been distinguished using a morphological species concept (MSR) or the biological species concept (BSR) (Zhang et al. 2010). The study of polymorphic characters like heterogeneity, identity and variability among the nucleotide sequences are assessed using the FINGERPRINT server (Kakoti et al. 2021).

Whole genomic sequences are the initial phase of molecular investigation in order to check species identity and biological properties but has its own problems. Random amplified of polymorphic DNA (RAPD) is the primary DNA based taxonomic tool that scans the complete genome of an organism in single reaction (Linacre, A, Cole & Lee 2002). All herbarium collected specimens is prepared and voucher specimens created for use in RAPD fingerprinting. RAPD fingerprinting has been found to be useful for the determination of species of Phytophthora (Boekhout, Toen et al. 2002) and may be useful for this genus (*Psilocybe*) as well. In addition, molecular genetics is used to assist in the differentiation of species. The identification of fungi by means of a DNA- based

test, which can be used in combination with morphological features, assists us to be assure of our identification (Lee, Cole & Linacre 2000b).

RAPD is a low-cost, simple to use and involves little in the way of equipment. The specific RAPD can assembly members of the same fungal species when compared to other fungal examples from a diversity of linked species from the same and anther genus. This technique is not trustworthy (Linacre, A, Cole & Lee 2002). The RAPD fingerprinting scheme is most frequently used for the perception of populations. RAPD analysis from *Psilocybe* discovered an overall large diversity (Boekhout, Toen et al. 2002).

Amplified fragments length polymorphism (AFLP) combines PCR with the use of restriction enzymes. It has rapidly become a standard tool in taxonomy and evolutionary studies. Similar to RAPD no prior sequence data is required. The first step is to digest all DNA with a combination of restriction enzymes that will end result in double stranded DNA fragments with single stranded overhangs of DNA due to the restriction enzymes. These overhangs perform as anchors to which small fragments of known DNA sequence are attached. These known attachments do as the priming site in PCR to amplify the fragments of DNA formed by the restriction sites. The resultant band patterns are detached on gels to produce a band pattern which is comparable to RAPD.

Due to the selective nature of the restriction enzymes, the band pattern is far further reproducible than RAPD. This procedure can like members of the same species and cluster members of the same genera. AFLP will group more efficiently all members of the same species and show them to be more connected to each other, when compared to a sample from an unlike species. The technique does not group all members of the same genus but inside a closed defined group species identification is promising. The disadvantages with this method are that, it needs 250ng of high molecular weight DNA and it takes up to three days to complete. RAPD and AFLP study the entire genome with AFLP being the more reliable and reproducible method and produce a classic DNA fingerprint (Linacre, A, Cole & Lee 2002).

NGS can capture low abundance DNA and provide detailed analyses of relative abundances in microbial communities at a relatively low cost. Among NGS

methods, Illumina MiSeq sequencing (Lower-throughput, bench-top version sequencing machine) is the most effective and extensively used technology globally due to its low rate of error and the lowest cost per Million bases (Mb), but requires short diagnostic regions of 300 base pairs to be effective. This method uses Genome specific DNA Sequencing inside the Internal Transcribed Spacer of the Ribosome gene compound (Lee, Cole & Linacre 2000b).

Pre-NGS steps: Sample preparation, DNA Extraction, PCR amplification, Confirmation of amplification (gel or capillary electrophoresis), PCR clean-up (Meier et al. 2016) and finally direct sequencing. The use of molecular genetics is used to assist in the differentiation of species to a higher level of resolution (Maruyama et al. 2006) (Linacre, A, Cole & Lee 2002). At present, it is envisioned that, the use of NGS (Next Gene Sequencing) will be the most effect molecular analysis tool for differentiation of species as other methods are not as refined nor easily applied (Tsujikawa et al. 2003).

DNA barcoding is an efficient method to identify specimens and to detect undescribed / cryptic species. Sanger sequencing of individual specimens is the standard approach in generating large-scale DNA barcode libraries and identifying unknowns. However, the Sanger sequencing technology is, in some respects, inferior to next-generation sequencers, which are capable of producing millions of sequence reads simultaneously.

Additionally, direct Sanger sequencing of DNA barcode amplicons, as practiced in most DNA barcoding procedures, is hampered by the need for relatively hightarget amplicon yield, coamplification of nuclear mitochondrial pseudogenes, confusion with sequences from intracellular endosymbiotic bacteria (e.g. *Wolbachia*) and instances of intraindividual variability (i.e.heteroplasmy). Any of these situations can lead to failed Sanger sequencing attempts or ambiguity of the generated DNA barcodes. Here, we demonstrate the potential application of next-generation sequencing platforms for parallel acquisition of DNA barcode sequences from hundreds of specimens simultaneously. The process of DNA typing and DNA profiling employed depends upon the facts known about the species or sample in question (Linacre, A, Cole & Lee 2002).

The IMS methodology has to date been successfully utilized for the detection of prescription and illicit drugs as well as for the detection of cocaine, methamphetamine and other exclusive drugs. Work carried out in this study will involve both morphology and molecular components that focus on the identification and characterisation of taxa (species) of fungi. Even though the possible use of the compounds found in the genus *Psilocybe* have the potential for more widespread use and application, the development of these useful compounds is limited by the reliance on wild-source materials that lack quality control and full characterisation of their chemical profiles and concentrations. The methods developed and molecular / chemical 'fingerprints' identified in this study will allow future researchers to quickly and easily identify taxa that are unknown to them. The approach of combined methods consists of molecular and morphology detection with the influence of phylogenetic analysis seem to be a promising tool in order to ambiguously identify this taxa (Nugent & Saville 2004).

10.6 Primer Design (Choice of Marker)

The existence of universal primers with sufficient taxonomic resolution is one of the main criteria required for a universal barcode in fungi. Protein coding genes are widespread phylogenetic markers in mycology and they are used as *de facto* barcodes of partial taxonomic scope in several groups of fungi. In overall such protein markers had more species resolving power, but PCR and sequencing failures exclude them as potential universal barcodes for the broad phylogenetic scope of the kingdom fungi (Schoch et al. 2012). Nevertheless, the function of DNA as a tool to catalogue biodiversity resolve phylogenies or discover outlines in ecological communities depends powerfully on choice or enterprise of primers for choosing the right genetic markers. Primer sets need to be universal enough to match across all members of a wide taxonomic group whereas containing mismatches to nontarget taxa and so far, yield a gene product, flexible enough to distinguish taxa at slight rather species level resolution (Asemaninejad et al. 2016). The use of a short DNA region to delineate and isolate species has been formalized under the term barcoding for even shorter fragments (Ryberg 2015).

Relative metagenomics studies of closely related species, phylogenetic signal and genetic changeability of mitochondrial genes may agree the discovery of more useful mitochondrial genes and better indicative markers and have the potential to provide better nodal support for phylogenetic relationship inference. Latest studies on the comparative nuclear genomics moreover offer the promise of finding supplementary independent loci that may capture genetic difference at diverse scales to be used in combination with already well-established ribosomal and mitochondrial genes. Introns of single copy genes are perfect for fine-scale studies (population or intraspecific level variation) since they have little structural constraint, while neighbouring exons are archetypally conserved, making it possible to design PCR primers. Such markers, habitually called EPIC (exon-primed intron-crossing) can be defined with increasing easiness now, that complete genome sequences are accessible for numerous species and other closely connected taxa (Blasco-Costa et al. 2016).

The existence of universal primers with sufficient taxonomic resolution is one of the major criteria required for a universal barcode in fungi. The lack of a universally recognized DNA barcode for fungi, the second most species eukaryotic kingdom is a serious limitation for multitaxon ecological and biodiversity studies. DNA barcoding uses standardizes 500- to 800- bp sequences to identify species of all eukaryotic kingdoms, using primers that are valid for the largest potential taxonomic group. Reference barcodes must be derived from skilfully identified vouchers deposited in biological collections with online metadata and certified by existing online sequence chromatograms. Interspecific variation should go beyond intraspecific variation and barcoding is optimum when a sequence in constant and matchless to one species. Perfectly the barcode locus should be equal for all kingdoms (Schoch et al. 2012). Highly variable lengths and high evolutionary rates for the nuclear ribosomal cistron in species of Basidiomycota may provide challenges for sequencing and analysis. In this study, we perform a large-scale analysis of all the available *Psilocybe* and *Deconica* sequences from GenBank. We carry out a rigorous trimming of the initial dataset based in methodological principals of DNA Barcoding. Knowledge regarding the efficiency and limitations of the barcode markers that are currently used for the identification of organisms is crucial because it benefits research in

many areas. Our study provides information that may guide researchers in choosing the most suitable genomic markers for identifying Basidiomycota species. We investigate the diversification of the 96 samples to classify them by a genetic approach as well as traditional morphology technique. In this project, we describe the phylogeny of species of *Psilocye* based on the partial sequence of these three regions. To avoid confusion, an epitype collection characterized by molecular data of following designated primers (ITS rDNA, LSU, EF-1a) give the most reliable results. The best genetic marker would have high inter- and low intra-species sequence variance, i.e., a discrete barcode gap (Stielow et al. 2015).

Extremely valuable length and high evolutionary rates for the nuclear ribosomal cistron in species of Basidiomycota might offer trials for sequencing and analysis. DNA barcoding uses standardized 500- to 800-bp sequences to identify species of all eukaryotic kingdoms using primers that are applicable for the broadest possible taxonomic group. Reference barcodes must be derived from expertly identified vouchers deposited in biological collections with online metadata and validated by available online sequence chromatograms barcoding is optimal when a sequence is constant and unique to one species (Schoch et al. 2012).

In this study we report on the isolation and complete sequencing of the three regions from members of the fungal genera *Psilocybe*. Due to the importance of this species, in which is considered as a major medicinal species, genetic diversity of its isolates is assessed using three markers in particular. Among *Psilocybe* isolates, 96 isolates screened from different regions are selected. Many of the species share identical or nearly ITS rDNA alleles. However, satisfactory taxonomic identification remains problematic in the kingdom Fungi due to the vast, largely unexplored diversity and the lack of reliable and richly annotated reference sequences. Given the fungal kingdom's age and genetic diversity, it is unlikely that a single-marker barcode system will be capable of identifying every specimen or culture to species level. Many previous studies of fungal taxonomy have used similar DNA sequences of the nuclear ribosomal RNA repeat for determining relations at the genus and species levels (Nugent & Saville 2004).

The presence of universal primers with adequate taxonomic resolution is one of the main standards vital for a universal barcode in fungi (Větrovský et al. 2016). Primers specificity or the absence of primer universality in improving broad groups such as the whole kingdom fungi has been a persistent concern that has not formerly been addressed for biodiversity studies using the Illumina platform (Asemaninejad et al. 2016). Gene / locus designated for study of DNA barcoding in fungi are ITS, LSU (mitochondrial genome / ribosomal DNA) and EF (nuclear genomic DNA) (Purty & Chatterjee 2016). There are distinctive rewards in inspecting a certain polymorphic locus (Linacre, A, Cole & Lee 2002). We demonstrate how these primers can be applied for discovery of key fungal groups to recover a broad range of fungal taxa with confident higher-level placement (family, order, etc.) and potential species or species-group identification.

Although the protein-coding gene regions often have a higher percent of correct identification compared with ribosomal markers, low PCR amplification and sequencing success, eliminate them as candidates for a universal fungal barcode. Protein markers has more species resolving power, but PCR and sequencing failures, eliminate them as potential universal barcodes for the broad phylogenetic scope of the kingdom Fungi. These markers could properly separate these ecotypes based on geographical distribution and similarity in morphology and show the wide genetic diversity among this taxa. The markers are used in order to assess the genetic relationship between this taxa.

Molecular (DNA sequence) data have emerged as crucial information for the taxonomic identification of plant pathogenic fungi, with the nuclear ribosomal internal transcribed spacer (ITS) region being the most popular marker. The need for a second marker depends on the intended purpose of an investigation. However, international nucleotide sequence databases are accumulating numerous sequences of compromised or low-resolution taxonomic annotations and substandard technical quality, making their use in the molecular identification of plant pathogenic fungi problematic.

Markers can be targeted by well-stablished PCR techniques on novel hybridization techniques and consequently combined for high-throughput sequencing using parallel-tagged sequencing of multiple distinct samples within a

single sequencing run (Leavitt, Moreau & Thorsten Lumbsch 2015). The leading issues when using molecular methods in taxonomic studies are (i) which and how many markers should be used; and (ii) how should they be analysed? First, one must consider 'What are the disadvantages of the chosen gene(s)?' As different genes evolve at unlike rates, choosing the optimal genetic marker, necessitates cautious consideration of the aim of the study. All genes accumulate mutations over time but some experience higher rates of genetic change (i.e., those unconstrained by function), whereas others accumulate fewer mutations (i.e., those encoding for particular biological functions). The challenge is to attain an equilibrium. A chosen gene must show sufficient variant to distinguish closely related taxa but not so much as to hinder strong taxonomic / systematic / phylogenetic inferences. Typically, the most suitable markers are selected based on prior knowledge for the family of genus being studied and are those that exhibit 70% but less than 100% similarity (BlascoCosta et al. 2016).

Metabarcoding refers to the rapid biodiversity assessment that combines DNAbased species identification and high-throughput DNA sequencing. Fungal Metabarcoding uses universal PCR primers to mass-amplify DNA barcodes from mass collections of organisms or from environmental DNA (eDNA). The use of short uniform DNA sequences or DNA barcodes for the purpose of singular identification of organisms has contributed to diverse zones of biological investigation (Shokralla et al. 2014). It is extensively known that use of degenerate primers can impressively reduce the specificity of PCR amplifications. When using the degenerate primers sequence quality is poor (Větrovský et al. 2016).

Fungal barcoding, that is the use of genetic markers to classify fungal species, has contributed massively to the rise of mycorrhizal research in the last years. Because it allows fast and easy identification of species or developed taxonomic levels and grouping of sequences into units; this speed up ecological analysis and the discovery of new species. Marker sequences agree the delineation of operational taxonomic units (OTUs) on the base of sequence similarities. Particular cases of OTUs delineated through datasets, are internal transcribed spacer (ITS) based fungal species theory and small subunit (SSU) rRNA

(Selosse, Vincenot & Öpik 2016). Although the spectrum of the most abundant OTUs identified by both primer sets is oddly overlapping their abundance contrasted fundamentally (Větrovský et al. 2016).

The region that separates the cluster of three genes along the chromosome is called the non-transcribed spacer (NTS) and prior to where the 18S gene is transcribed, there is another small spacer region called the externally transcribed spacer (ETS). Together the ETS and NTS regions include the intergeneric spacer region. These constituents are repeated in a tandem array, but they develop as a single unit and disagree in length around 3000–4500 base pairs. The ribosomal DNA has attracted increased attention among fungal systematists, specifically those interested in relating DNA sequencing analysis to study taxonomic relationships and genetic deviation in fungi. The utmost notable feature of the rDNA is the general sequence homogeneity among repeat units of the gene family. This gene shares the same function in all organisms and develops at roughly the same ratio. However, the three different regions (structural genes, transcribed spacers, NTS) evolve at dissimilar rates, therefore yielding informative data to rebuild the phylogeny at different taxonomic levels (Jeewon & Hyde 2007). Certainly, a mutation in one repeat or a meiotic crossing over within the rDNA locus can result in heterogeneous repeats on the equivalent chromosome as observed in some fungi. But in most circumstances a mechanism named concreted evolution acts to homogenize the repeats within a chromosome (Selosse, Vincenot & Öpik 2016).

Some specific terms; Hairpin

A hairpin loop is formed when primer folds back upon itself and is held in place by intramolecular bonding. Because hairpin loop formation is an intramolecular reaction, it can occur with as few as 3 consecutive homologous bases. To measure the stability of the hairpin loop formed, measure the free energy.

Dimers and Cross Dimers

Dimers occur when a region of homology is present within a primer (self -dimer) or between the sense 9 and anti-sense primer (cross dimer). This results in

bonding of the two primers, increasing production of the primer dimer artifact and reducing product yields.

Dimers occur within a primer when two copies of the primer bind to each other and cross dimers occur when a primer binds to the other primer in the pair.

3' end stability

The stability of the primer determines its false priming efficiency. An ideal primer has a stable 5' end and an unstable 3' end.

If the primer has a stable 3' end, it will bond to a site which is complementary to it other than the target with its 5' end hanging off the edge. It may then lead to secondary bands. Primers with low stability at the 3' ends, function well because the 3' end bonding to false priming sites are too unstable to extend. Repeats and Runs increase the likelihood of false priming.

5′– [Illumina Forward Adaptor] [NNNN] [Barcode] [PCR Primer]–3′

Primers are ordered with an Illumina adaptor linker and unique barcode as described above. Both forward and reverse primers were modified on their 5 ends to include the following forward and reverse Illumina Miseq adaptors to agree sequences to bind to the flow cells a 4 bp random linker (NNNN) to increase the diversity for the first loci sequenced and therefore aid the instrument to more effortlessly isolate clusters and set correct fluorescence levels and irreplaceable 8 nt barcode sequences with an edit distance of at least 4 (Asemaninejad et al. 2016).

Most of the studies on the Ascomycota and the Basidiomycota have reported a within-genome variability lower than 3%, which corresponds to the ITS rDNA is a quantitatively dominant marker in public databases, followed by b-tubulin (tub2) or actin, translation elongation factor 1-a (tef1a), and the second largest subunit of ribosomal polymerase II (RPB2) (Feau et al. 2011) and the last two also represent the markers included in the Assembling the Fungal Tree of Life (AFTOL) mission (Větrovský et al. 2016).

ITS rDNA is a quantitatively leading marker in public databases followed by b tubulin, actin, translation elongation factor 1-x (1-α) and the subsequent largest

subunit (RPB2) and the last two likewise represent the markers included in the assembling the fungal Tree Of Life (AFTOL) mission. Of them tef1-x is not universally existent in fungi and together with tub2 is known to have paralogou s copies in certain fungal genomes. Thus, the RPB2 gene is a fit alternative marker characterized in 6378 from 30780 species deposited in National Centre for Biotechnology Information (NCBI) GenBank. ITS rDNA was planned as the widespread fungal barcode for its interspecific variability and the obtainability of conserved primer locates. Yet its intragenomic variability could represent a likely limitation for the study of fungi from environmental DNA. Cloning or enormously analogous sequencing unlike Sanger sequencing from genomic DNA yields sequences derived from single alleles and fungal miscellany may be considerably overrated if these sequences are satisfactorily different within a fungal individual or taxon. The variable length of ITS region represents one more vital problem because there is a robust PCR bias against species with longer amplicons a matter that was found to mainly disturb the outcomes of community studies. Finally, the low conservancy of the ITS region stops its usage in phylogenetic studies on higher taxonomic orders. The protein encoding genes are suitable for this as the translated amino acid sequences can be used for high quality alignments of unrelated fungi. This could be remarkably valuable for the proper placement of unidentified higher taxonomic level lineages which are repeatedly faced in environmental samples. For the above details the use of partial sequence data of the single-copy protein encoding genes as alternate markers could be an answer to the multiple complications accompanying with rDNA-based markers including the RPB2 gene have a superior species resolving power than rDNA markers the lack of universal primers is supposed to limit their use as potential barcodes (Větrovský et al. 2016). Nuclear and mitochondrial ribosomal RNA loci made accessible by universal primers.

DNA sequence analysis of the internal transcribed spacer (ITS), including the 5.8S region, the large subunit nuclear ribosomal RNA (nLSU), translation elongation factor 1-alpha (tef1-α) and the second largest subunit of RNA polymerase II (RPB2), two non-protein coding (ITS and nLSU) and two protein coding genes (tef1-α and RPB2). The large subunit of the nuclear ribosomal RNA (LSU), a favoured phylogenetic marker among many mycologists, had virtually no

amplification, sequencing, alignment or editing problems and the barcode gap is superior to the Small Subunit of rRNA (SSU). However, across the fungal kingdom, ITS is generally superior to LSU in species discrimination and had a more clearly defined barcode gap. ITS have been reported to perform as a close second to RNA polymerase II largest subunit (RPB1) as the protein-

coding marker. However, the much higher PCR amplification success rate for ITS may pose a critical difference in its performance as a barcode.

The strongest sign of a barcode gap is seen for RPB1 followed by ITS LSU and SSU performed poorly each missing a noteworthy barcode gap. The PCI of ITS is slightly lower than RPB1. RPB1 constantly yielded high levels of species discrimination in all of the fungal collections apart from the early diverging lineages which is equivalent with multigene combinations. ITS had the most resolving influence for species discrimination in Basidiomycota. ITS had lower discriminatory power than SSU and LSU in primary diverging lineages but boundaries of fault are high. LSU had variable levels of PCI among all groups but is usually lower than RPB1. In Sacharomycotina LSU has the lowest PCI but all four markers perform equally. SSU is consistently the worst performing marker with the lowest species discrimination in Basidiomycota. In the early diverging lineages SSU has a better PCI on balance with LSU and better than both ITS and RPB1 (Schoch et al. 2012).

About 80% of respondents reported no problems with PCR amplification of ITS, 90% scored it as easy to obtain a high-quality PCR product, and 80% reported no significant sequencing concerns. In comparison, >70% reported PCR amplification problems for RPB1; 40–50% reported primer failure as the biggest problem. (i.e., ITS, LSU, SSU, and RPB1). SSU is consistently the worst performing marker, with the lowest species discrimination in Pezizomycotina and Basidiomycota. In the early diverging lineages, SSU had a better PCI, on par with LSU and better than both ITS and RPB1.

The highest-ranked three- and four-gene combinations gave comparable increases. The clearest indication of a barcode gap is seen for RPB1 followed by ITS. LSU and SSU performed poorly, each lacking a significant barcode gap. Overall, ribosomal markers have fewer problems with PCR amplification than

protein-coding markers. Phylogenetic marker amongst many mycologists has virtually no amplification sequencing alignment or editing problems and the barcode gap is superior to the small subunit of rRNA. Yet through the fungal kingdom ITS is commonly superior to LSU is species discrimination and has a more undoubtedly defined barcode gap. ITS have been stated to do as a close second to RNA Polymerase II largest subunit (RPB1) as the protein-coding marker. However, the much higher PCR amplicon success level for ITS might pose a critical variance in its performance as a barcode as well as a range of biases for all primer sets, therefore an appropriate solution may be to use more than one primer combination (Purty & Chatterjee 2016).

The eukaryotic rRNA cistron consists of the 18s, 5.8 s and 28s rRNA genes transcribed as a unit by RNA polymerase I. posttranscriptional processes divided the cistron eliminating two ITS. These two spacers including 5.8s gene are typically mentioned as the ITS region. The 18s nuclear ribosomal small subunit rRNA gene (SSU) in normally used in phylogenetic and while in homologs (16s) is frequently used as a species diagnostic for bacteria it has less hypervariable domains in fungi. The 28s nuclear ribosomal large subunit rRNA gene (LSU) occasionally discriminates species on its own or united with ITS. For yeasts the D1/D2 region of LSU is accepted for characterizing species lineage before the conception of DNA barcoding is encouraged. Presently nearby 172,000 full lengths fungal ITS sequences are deposited in GenBank. For those who opt to query GenBank, we recommend the use of longer sequences, rather than shorter sequences (O'Donnell et al. 2015). In a reduced number of environmental studies, ITS has been used mutual with LSU. ITS is likewise used in some fungi for providing a sign of delimitation by a measure of genetic distances. Conversely phylogenetic methodologies are also being used to identify taxonomic units in environmental sampling for fungi and are more operative in comparison. Protein coding genes are broadly used in mycology for phylogenetic analysis or species identification. For Ascomycota they are commonly superior to rRNA genes for determining relationships at various taxonomic levels (Schoch et al. 2012).

ITS and LSU perform very similarly as barcodes and that alterations in these sequences correlate well with existing species perception (Schoch et al. 2012). The ITS and LSU rRNA genes are more common in taxonomic research

(Selosse, Vincenot & Öpik 2016). There is general promise at higher taxonomic levels between the fungal communities detected using the LSU primers and ITS2 primers (Asemaninejad et al. 2016). Such protein markers have more species resolving power, but PCR and sequencing failures eliminate them as potential universal barcodes for the broad phylogenetic range of the kingdom Fungi. ITS and LSU perform very equally as barcodes and that differences in these sequences correlate well with current species concepts. If the taxa are taxa with low ITS interspecific changeability, secondary markers must be used to truthfully report genetic diversity one or any combination of the six genes (Schoch et al. 2012) but the need for a second marker depends on the intended purpose of an investigation.

For certain groups of fungi, additional sequencing of the nLSU region may not suffice for accurate species differentiation. In some species of fungi that are difficult to differentiate using the ITS and nLSU regions, full identification is performed only by analysis of sequences of genes encoding RNA polymerase (RPB1 and RPB2) in combination with sequences of the ITS region the ITS region is doubtful, it would be advisable to use supplementary markers (e.g., LSU, RPB1, and RPB2) (Kowalczyk, M et al. 2015). Of them, tef1a is not universally existent in fungi and together with tub2 is known to have paralogous copies in certain fungal genomes. Thus, the RPB2 gene is a suitable alternate marker characterized in 6378 from 30 780 species deposited in NCBI GenBank. ITS rDNA was proposed as the worldwide fungal barcode for its interspecific variability and the availability of conserved primer sites. Conversely, its intragenomic variability could represent a potential limitation for the study of fungi from environmental DNA (Větrovský et al. 2016).

To assess the PCI, data are divided into subsets according to taxonomic similarity. Within Dikarya, ITS has the most resolving power for species discrimination in Basidiomycota. For Pezizomycotina, the PCI of RPB1 outperformed ITS. ITS has lower discriminatory power than SSU and LSU in early diverging lineages, but margins of error are high. LSU has variable levels of PCI among all groups but is normally lower than RPB1 or ITS. SSU is constantly the worst performing marker, with the lowest species discrimination in

Pezizomycotina and Basidiomycota. In the early diverging lineages, SSU has a better PCI, on par with LSU and better than both ITS and RPB1.

In the multigene combinations, the most effective two genes in the combined analysis are either ITS and RPB1 or LSU and RPB1. This discovery represented a rise from the highest-ranked single gene. The highest- ranked three- and four-gene combinations offered comparable increases. The clearest indication of a barcode gap is seen for RPB1 followed by ITS (Schoch et al. 2012).

10.6.1 COI (Advantages and Disadvantages)

The region of the mitochondrial gene encoding Cytochrome c oxidase subunit 1 (cox1) used as the animal barcode recognized as a potential marker and the default marker accepted by the Consortium for the barcode of Life for all groups of organisms as well as fungi. But it is problematic to amplify in fungi and often includes large introns and can be inadequately variable. The de facto barcode, internal transcribed spacer (ITS) region is appropriate for identification, however the default COI marker is more dependable in a few clades of strictly associated species. COI carry out well in *Penicillium* and other fungi (Purty & Chatterjee 2016).

The nuclear rRNA cistron has been used for fungal diagnostics and phylogenetic for more than 20 years and its components are most often debated as alternate to COI. The eukaryotic rRNA cistron consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a unit by RNA polymerase I. COI functions reasonably well as a barcode in some fungal genera, such as *Penicillium*, with reliable primers and adequate species resolution. Because most fungi are microscopic and inconspicuous and many are unculturable, robust universal primers must be available to detect a truly representative profile. This availability seems impossible with COI and this system sets a precedent for reconsidering COI as the default fungal barcode. Thus robust, universal primers are required to spot a truthfully representative profile where, COI appears to have many challenges from other candidates like ITS. This system sets a standard for reassessing COI as the default fungal barcode. Since most fungi are microscopic and

inconspicuous and many are unculturable, robust general primers need to be accessible to identify a correctly typical profile. This obtain ability looks impossible with COI. Three DNA regions are evaluated as potential DNA barcodes for Fungi, the second largest kingdom of eukaryotic life, by a multinational, multi-laboratory consortium. The region of the mitochondrial Cytochrome c oxidase subunit 1 used as the animal barcode is excluded as a potential marker, because it is difficult to amplify in fungi, often includes large introns, and can be insufficiently variable.

The defacto barcode, internal transcribed spacer region is fit for identification, but the default COI marker is more trustworthy in a few clades of thoroughly correlated species (Schoch et al. 2012). Barcoding is optimal when a sequence is constant and irreplaceable to one species the de facto barcode internal transcribed spacer (ITS) region is suitable for identification, but the default COI marker is more reliable in a few clades of closely related species.

10.6.2 ITS (Advantages and Disadvantages)

Fragments encoding ribosomal RNA are separated by non-coding fragments, which are removed after the transcription phase. These so-called internal transcribed spacers (ITS1 and ITS2) and non-transcribed intergenic spacers (IGS) are characterized by the presence of polymorphisms of length and sequence, which make them greatly useful for species identification. ITS represents a vastly polymorphic marker and subsequently a powerful implement for taxonomic purposes at species level (Borovička et al. 2011). ITS-rDNA sequences are divergent and vary between species within a genus, therefore it is the most popular choice in molecular systematics (Rai et al. 2014). The rDNA locus contains tens to hundreds of tandem repeats, each of which encodes the four ribosomal RNA genes parted by spacers. Fragments encoding ribosomal RNA are separated by non-coding fragments, which are removed after the transcription phase. The nuclear internal transcribed spacer (ITS) ribosomal DNA gene region is the acceptable DNA barcode for fungi (Shokralla et al. 2014).

The ITS region consists of two non-coding nuclear DNA regions (ITS1, ITS2) (The ITS1 region + the ITS2 region =600-750 bp) that separate three ribosomal RNA genes (rRNA): 18S, 5.8S and 28S. On the terminals of this complex, there are external transcribed spacer (ETS) regions: ETS1 and ETS2, located correspondingly within the 5' and 3' terminal region. In fungal genomes, noncoding regions and nuclear ribosomal RNA genes (nrRNA) occur as numerous (60-220) tandem repeats. A large number of nrRNA gene replicas allow their amplification even in significantly degraded material. The high sensitivity of the analysis of ITS1 and ITS2 DNA sequences makes the regions a reliable marker in phylogeny classification. The ITS and IGS regions evolve faster and are extremely variable and so valued for comparing fungal species at the intraspecific level (Stielow et al. 2015).

Within each repeat the ITS is positioned between the small subunit (18s rRNA) and 28srRNA and embraces the 5-8 rRNA gene. The multi copy structure of rDNA eases its amplification by PCR and makes it very appropriate for taxonomic barcoding (Selosse, Vincenot & Öpik 2016). In fungi, the most important target region is nuclear DNA encoding ribosomal RNA (nrDNA), arranged in the form of tandem repeats. The number of copies of nrDNA genes in mushrooms varies from 60 to 220, depending on the species. The ITS1 region carries the extra benefit of having a priming site for the fungal-specific primer ITS1F (Osmundson et al. 2013). Sequence comparisons of designated regions within the rDNA have been suitable for assuming phylogenetic relationships among fungi for some reasons. Using specific DNA polymorphisms such as isolated in the ITS region of the two fungal genera, allows perfect sizing in method used normally (Linacre, A, Cole & Lee 2002). The best effective barcoding region in fungi is ITS for its high copy number, the accessibility of universal primers, the success rate of amplification and the highest variations compared to other loci. This barcoding region can differentiate even for intraspecific variant. Other loci, although afford highly fruitful amplification rates, they produce less variation than ITS (Kaewgrajang et al. 2020). Although not true for all fungal groups, for the great number of chiefly macro-fungal samples tested here, ITS1 is meaningfully superior to ITS2 in terms of species perception. The use of the ITS region for molecular identification of fungi goes back to the early 1990s. A small number of

genes have been used for molecular characterization of fungi and their phylogenetic analysis. In many cases, the protein-coding gene regions are found to have a higher percentage of rightness in identification compared with ribosomal markers. However, low PCR amplification and sequencing success are main disadvantages for their use as candidates for a worldwide fungal barcode. Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) has been an operative marker of choice for their resolution at intra- and interspecific level (Kakoti et al. 2021). But in fungi, this region (ITS) enables identification of only 20%-70% of species. Currently, ~ 172,000 full-length fungal ITS sequences are deposited in a smaller number of environmental studies, ITS is been used combined with LSU. ITS is also used in some fungi for providing an indication of delimitation by a measure of the genetic distances. However, phylogenetic approaches are also being used to identify taxonomic units in environmental sampling of fungi and are often more effective in comparison. Protein-coding genes are widely used in mycology for phylogenetic analyses or species identification. For Ascomycota (including mould genera such as Aspergillus), they are generally superior to rRNA genes for resolving relationships at various taxonomic levels (GenBank translation elongation factor 1-α). However continuing investigation of the ITS for fungal diversity has discovered that the ITS region is excessively variable to align over distantly correlated taxa and thus incapable to assuredly place sequences exist. Furthermore, in some genera of filamentous ascomycetes there is tiny variant in the ITS region making this dominion unwanted for identification or taxonomic analysis at the species level. General fungal identification and taxonomic analysis using ITS remain difficult. The D1 variable region of the large ribosomal subunit is an attractive alternate as it has confirmed worthwhile in species level identification and phylogenetic reconstruction in various fungal group (Asemaninejad et al. 2016). While the ordinarily used ITS region of rDNA is well matched for taxonomic identification of fungi, the information on the relative abundance of taxa and diversity is adversely affected by multicopy nature of rDNA and the existence of ITS paralogues. Furthermore, due to high variability, ITS sequences cannot be used for phylogenetic analysis of distinct taxa (Větrovský et al. 2016). Another main gain of using ITS as a barcode is that each haploid genome naturally contains multiple tandemly repetitive copies of the

ribosomal rRNA gene cluster (including ITS), making it probable to amplify this gene from small volumes of biological materials. The ITS region is better at differentiating narrowly allied species, than the D1/D2 domain. In addition, it is been found that the intergenic spacer (IGS, situated between the 3=-end of the 28S rRNA gene and the 5=end of the 18S rRNA gene) region of the ribosomal gene cluster could also be used for additional variation of species and even among strains within the same species. Because the ITS sequences normally show greater variant and have better discriminating control than the D1/D2 domain between thoroughly related taxa. ITS has the most resolving influence for species discrimination in Basidiomycota. ITS sequences are used to investigate fungal diversity, from specimen identification to exploring the spatial and temporal distribution of fungi in a diversity of ecological niches. Alike to that for pure specimen studies, using ITS sequences to analyse environmental fungal variety is applicable (James 2015).

The enclosure of at least one conserved region (18S or 28S rDNA) and one spacer (ITS1, ITS2, or the entire ITS1-5.8S-ITS2 region) for all new species explanations is recommended. This will offer an inclusive database for future delineative studies (Blasco-Costa et al. 2016). The ITS region is maybe the most extensively sequenced DNA region in fungi. This region has higher degree of deviation than other genetic regions of rDNA and are polymorphic, so provide sequence variability that allows distinguishing among different species or strains of mushrooms. The ITS region has the top likelihood of effective identification for the broadest range of fungi, with the most openly defined barcode gap between inter- and intraspecies variation (Hussein et al. 2014). The rDNA gene sequences can be derived with Internal Transcribed Spacer (ITS) and nontranscribed intergenic spacer (IGS) (Lee, Cole & Linacre 2000a).

The ITS 1 and ITS 2 markers are used universally in fungi taxonomy (Zuber, Kowalczyk, Sekula, et al. 2011). ITS2 have been magnificently used for the verification of narrowly connected species (Purty & Chatterjee 2016). Two different approaches (PCI and barcode gap) are used to determine the performance of the complete ITS region and sub-regions. Internal transcribed spacer (ITS) region is phylogenetically beneficial for assessment of recently differed fungal groups. The complex phylogenetic relationships and the presence

of cryptic species in some genera are possible explanations of the ITS limitation (Leavitt, Moreau & Thorsten Lumbsch 2015).

The internal transcribed spacer region (ITS) is played a principal part in molecular phylogenetic studies of lichenized fungi and is newly been accepted as the official barcoding marker for fungi in numerous cases. The ITS is adequately variable to resolve species margins for lichenized fungi. Although precise specimen identification, using sequence-based identification methodologies call for a carefully curated reference database in spite of the universal function of the ITS marker, a number of matters may theoretically limit its operative use in species delimitation studies plus the potential for intragenomic deviation of nuclear ribosomal tandem repeat and complications in aligning ITS sequences from divergent taxa. Since the ITS is been officially implemented as the formal barcoding marker for fungi, we mention that species delimitation studies try to include this region for reliability across studies. Conversely, we spot that in some taxonomic groups, the inclusion of the ITS may be challenging and then recommend a cautious screening of other markers to identify correct loci for solving species-level relationships. Eventually the success of any single-locus species delimitation method, mainly depends on the evolutionary history of the species group under study and the variability of the designated marker (Leavitt, Moreau & Thorsten Lumbsch 2015).

The Ribosomal Internal transcribed spacer (ITS1 and / or ITS 2 rDNA) are the leading fungal barcodes (Purty & Chatterjee 2016). Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest likelihood of effective identification for the broadest range of fungi with the most obviously defined barcode gap between inter- and intraspecific variation. The nuclear ribosomal large subunit, a popular phylogenetic marker in certain groups has superior species resolution in some taxonomic groups such as the primary diverging lineages and the ascomycete yeasts, but is then marginally inferior to the ITS. The nuclear ribosomal small subunit has poor specific-level resolution as the chief fungal barcode marker to the Consortium for the barcode of life with the chance that additional barcodes may be settled for particular hardly circumscribed taxonomic group. In an expanded dataset of ITS sequences from our fungal DNA barcoding database, the highest variant is most often found in

the early diverging lineages. ITS combines the peak resolving power for discriminating strictly related species with a high PCR and sequencing success rate across a broad range of fungi (Schoch et al. 2012).

In molecular systematics, ITS rDNA sequencing is the most reliable tactic for the fungal identification to species level. There are cases where two or more taxa that are morphologically and ecologically diverse, cannot be distinguished based on ITS rDNA marker. In addition, sequencing of the ITS region does not agree for individualization of samples, as it is extremely conserved within a species (Kallifatidis et al. 2014).

There are primers helping in the amplification of both ITS regions, including the 5,8S coding region, as well as species- specific primers pointing the ITS, shows that hallucinogenic mushrooms from the genera *Psilocybe and* some others can be differentiated on the basis of differences in the lengths of ITS1 PCR products. Analysis of the sequences of ITS regions is valuable for creating molecular systematics at the species level, and even for describing intraspecific geographic distinction. Markers regularly employed in species identification of fungi are internal transcribed spacers regions (ITS1 and ITS2). The ITS1 and ITS2 regions are generally employed markers used in fungi species identification. ITS fragments are exceptionally valuable in species identification due to the presence of length and sequence polymorphisms. The use of the ITS4B primer is specific for the Basidiomycota (Zuber, Kowalczyk, Sekuła, et al. 2011).

The ITS2 primers which are designed to recover Ascomycota and Basidiomycota will not yield sequences of Chytrids, Glomeromycota or the Zygomycete groups. Studies directed on the polymorphism of the ITS region, encompassing mushrooms fitting many taxa, have shown the helpfulness of these regions as a simple universal marker in the Fungi kingdom. Primers complementary to the ITS1 region (primers ITS1F and ITS2) and ITS2 region (primers ITS3 and ITS4) are originally used for amplification. The research shows that sequencing of the ITS2 fragment is satisfactory in most cases and that it properly identified the most frequent causes of mushroom poisonings, as well as the most common hallucinogenic mushrooms (Asemaninejad et al. 2016).

Other studies show that the high degree of polymorphism of sequences of the ITS region may lead to uncertain identifications of strictly related species.

Studies carried out to date have revealed that the ITS regions yield excellent results in molecular systematics at the species level, as well as in the determination of interspecies geographical distinction. The usefulness of ITS regions polymorphisms analyses in species identification for categories purposes has been confirmed (Zuber, Kowalczyk, Sekuła, et al. 2011). Together, the LSU primers recovered some genera and species that are not obtained using the ITS2 primers, but the ITS2 primers recovered some unique genera and species that are not obtained, using either of the LSU primers. There is broad agreement at higher taxonomic levels between the fungal communities detected using the LSU primers and ITS2 primers. The nLSU rRNA data assemble isolates from the same species and separate hallucinogen containing and non-hallucinogen containing isolates into distinct clades. Many fungal taxonomy studies have used DNA sequence of the non-coding internal transcribed spacer (ITS) regions (locus 1 and 2) of the nuclear ribosomal RNA repeat for solving relations at the genus and species levels. The ITS-based identifications appear superior to those based on multi-locus analysis and excluded ITS. In fungal taxonomy research, uncertainties in phylogenetic analysis using the ITS-1 make species level discrimination problematic with the idea of approving. Resolution of phylogenies in these cases is often possible, using the nuclear large subunit of rRNA (nLSU, 28S). This region is less variable than the ITS-1 region and is readily amplified from a large group of Basidiomycete fungi. The sequences of the ITS and nLSU regions are determined using populations of amplified products from two or more independent amplifications. The data shows that combining analyses of the ITS1 and nLSU sequence variant has the resolving power to differentiate very closely species and separated hallucinogenic and non-hallucinogenic *Psilocybe* species (Nugent & Saville 2004).

A phylogenetic method for fungal identification to the level of species is using all variable characters in the ITS sequence. It has been suggested that a sequence database of the ITS and the first 900 bp of the divergent nLSU region would offer a reliable and automatic approach to the identification of hallucinogen containing

fungal isolates. In most of these conditions, barcoding regions such as the ITS may not be the most correct and it may be better to use for example the 5 ends of the large subunit of ribosomal DNA in a phylogenetic context or to try to measure the trait or function more straight for example by enzyme activity or using meta transcriptomics (Ryberg 2015).

While the usually used internal transcribed spacer region of rDNA (ITS) is well fitted for taxonomic identification of fungi, the information on the relative abundance of taxa and diversity is adversely affected by the multicopy nature of rDNA and the reality of ITS paralogues. Furthermore, due to high variability, ITS sequences cannot be used for phylogenetic analyses of unconnected taxa. Since the first mycological study applying NGS, parts of the rDNA cluster have been absolutely used as a molecular marker. The ITS rDNA region, reachable with universal primers, can adequately differentiate between most fungal species and is also the most abundant fungal marker in public databases. The rDNA cluster is a multicopy marker present in fungal genomes in 1–200 copies; even though the multicopy nature of this sequence eases obtaining ITS amplicons from low-quality DNA, it also utterly limits its value for the quantification of the relative abundance of fungal taxa. The multicopy nature also results in intraspecies and intragenomic variability. Comprehensive studies mapping the distribution of intragenomic ITS rDNA inconsistency among fungi is missing, although its extent is usually considered to be low with the exception of the Glomeromycota. The variable length of the ITS region represents another significant problem because there is a strong PCR bias against species with longer amplicons, an issue that is found to mostly affect the results of community studies. Finally, the low conservancy of the ITS region excludes its use in phylogenetic studies on higher taxonomic ranks. The protein-encoding genes are suitable for this, as the translated amino acid sequences can be used for high-quality alignments of unrelated fungi. This could be exceptionally valued for the appropriate placement of unknown higher taxonomic level lineages, which are regularly met with environmental samples for the above reasons. The use of single-copy protein encoding genes as alternative markers could be a solution to the multiple problems associated with rDNA-based markers. The ITS data set may

hypothetically contain deeper intragenomic paralogues, which increased number of singletons and OTUs in the ITS data set (Větrovský et al. 2016).

Although the protein-coding gene regions often had a higher percent of correct identification, compared with ribosomal markers, low PCR amplification and sequencing success eliminated them as candidates for a universal fungal barcode. Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest possibility of fruitful identification for the widest range of fungi, with the most obviously definite barcode gap between inter- and intraspecific deviation. ITS is formally suggested for adoption as the primary fungal barcode marker to the Consortium for the Barcode of Life, with the probability that complementary barcodes may be developed for particular narrowly circumscribed taxonomic groups. Protein-coding genes are widely used in mycology for phylogenetic analyses or species identification. For Ascomycota (including mould genera such as Aspergillus), they are usually superior to rRNA genes for resolving relationships at various taxonomic levels (GenBank translation elongation factor 1-α). However, the much higher PCR amplification success rate for ITS is a critical difference in its performance as a barcode. ITS primers are applied to a range of fungal lineages, and several primers function as almost universal primers. However, all primer sets have a range of biases, and a proper resolution will be to use more than one primer combination. Taking all these arguments into account, ITS is been advised as the standard barcode for fungi. The scheme will satisfy most fungal biologists but not all. Given the fungal kingdom's age and genetic diversity, it is unlikely that a single-marker barcode system will be capable of identifying each specimen or culture to species level. In an extended dataset of ITS sequences from fungal DNA barcoding database, the maximum variation is most often originated in the early diverging lineages. ITS combines the peak resolving power for discriminating faithfully related species with a high PCR and sequencing success rate through a broad range of Fungi (Schoch et al. 2012). ITS have been reported to perform as a close next to the most profoundly sampled of protein coding markers, RNA polymerase II largest subunit (RPB1). However, the much higher PCR amplification success rate for ITS may pose a serious difference in its performance as a barcode (Purty & Chatterjee 2016). Taking all these arguments into account, it is been recommend

ITS as the standard barcode for fungi. Assumed the fungal kingdom's age and genetic assortment, it is doubtful that a single marker barcode system is capable of identifying every specimen or culture to species level (Schoch et al. 2012).

The overall probability of correct species identification using ITS is comparable with the success reported for the two-marker plant barcode system. Species identification is problematic and time consuming even for those with special training and knowledge in medical mycology hence it becomes routine in many centres to sequence the ITS region of the rDNA for species identification. The rDNA of fungi exist as multiple copy gene family included of extremely similar DNA sequences (from 8 to 12 kb each). The ITS region of the rDNA is the most commonly sequenced DNA region in fungi. ITS is naturally most beneficial for molecular systematics at the species level and even intra species. This is because ITS characterized by high degree of deviation than other regions of rDNA such as small subunit (SSU) and large subunit (LSU) of the rDNA (Ahmed 2016).

The ITS /1F and other ITS primers have shown biases towards the amplification of fungal groups within the Basidiomycota, whereas other principally ITS2, ITS3 and ITS4 favour Ascomycota. More definite primers such as ITS4-B considered to capture Basidiomycetes can amplify the ITS region of a very restricted group within the target phylum (Asemaninejad et al. 2016). Mycorrhizal fungal research often uses the nuclear ribosomal ITS, now the most acknowledged taxonomic barcode for fungi (Selosse, Vincenot & Öpik 2016). The ITS and large subunit (LSU) rRNA genes are more common in taxonomic research. ITS and LSU perform very similarly as barcodes and that differences in these sequences correlate well with current species concepts (Liu 2016). Amplicon sequencing of the ribosomal ITS region and the entire genome sequencing of fungal isolates can be used as the most discriminative line in genetic study of various fungi (Zoll et al. 2016).

The Mendelian behaviour of rDNA in spite of its repetitive configuration is allowed its usage as a marker for population genetics for instance ITS polymorphism amongst characters of a population as a nonaligned marker to study population construction or the polymorphic intergenic spacer (IGS) between the 28s and 5s

rRNA genes to study Hardy Weinberg equilibrium. ITS sequencers are part of the ribosomal DNA locus that acts as a solo Mendelian locus. ITS based fungal OTUs are most ordinarily defined at the 97% sequence similarity threshold. Even if this likewise accounts for sequencing faults, it accepts the existence of its polymorphism within most fungal species. Analysing heterozygosity in ITS sequences of dikaryotic fruiting bodies shows how divergent the ITS region is in biological species of Basidiomycota. Species defined as a group of organisms capable of effective interbreeding. Most ITS sequences of Basidiomycota deposited in International Nucleotide Sequence Database (INSD) show no uncertain heterozygous positions. ITS sequences from single species samples such as fruiting bodies or fungal cultures are so far frequently obtained by Sanger sequencing directly applied to PCR products that is without a cloning step to separate diverse amplifications. Cloning is done when ampliWed products are either faint or displayed multiple bands (Selosse, Vincenot & Öpik 2016).

The nuclear ribosomal ITS region is the recognized barcode and in most cases the marker of choice for the investigation of fungal diversity in environmental models. Two problems are mainly critical in the search of satisfactory taxonomic project of recently produce ITS sequences: 1) the absence of a broad, trustworthy, public reference data set and 2) the lack of means to refer to fungal species for which no Latin name is accessible in a standardized stable technique. All fungal species represented by minimum two ITS sequences in the international nucleotide sequence databases are now agreed an irreplaceable unchanging name of the agreement number kind and their taxonomic and ecological annotations are modified as thinkable over a distributed third-party annotation effort. The ITS region has an elongated history of usage as a molecular marker for species level identification in ecological and taxonomic studies of fungi. It offers several benefits over other species level markers, in terms of high info content the formal barcode for fungi (Kõljalg et al. 2013). The use of DNA centred taxonomy to develop the characterization of fungal diversity is increasing. Newly a multi lab association exploring the top genetic marker for fungal taxonomy acknowledge the ITS region as the model marker gene database (Dannemiller et al. 2014).

ITS will then not be able to separate recently differing species in most cases and variances in communities established on ITS-MOTUs include processes performing on long period times. For ITS the concreted evolution of several replicas of the region in the genome is a noteworthy damage. There are cases, where the ITS region displays larger within species variant compared to between species distance than single replica genes. Additional studies are required to decide how well matched the union model is to model the evolution of the ITS region. The speciation rates, change rates, reasonable resident scope and generation times may be diverse in other organism groups than fungi and other barcoding regions than ITS. Countless studies have explored species complexes using crossing tests together with phylogenetic based on ITS sequences. There are also many instances where the largest within compatibility group ITS sequence distance larger than the least between compatibility group distances. For fungal ITS it would also necessitate that we resolve the problem of aligning the sequences. Generally used cut offs for ITS in fungi are unlikely to reveal units differing less than a couple of million years ago and differences in MOTU communities based on such cut offs are likely to be due to speciation interrelated with climate change during the Quaternary tales from the crypt (Ryberg 2015).

ITS sequences are amplified via PCR to allow the use of a sequencer Ion PGM for which the sequence length is restricted to nearly 300 bp at the time of sequencing (Izuno et al. 2016). Oligonucleotide primers for PCR amplification are planned to hybridize to the preserved regions of this, otherwise extremely inconstant locus (Nugent & Saville 2004). Most ITS sequences of

Basidiomycota deposited in INSD show no ambiguous, heterozygous loci. ITS sequences from single species samples such as fruiting bodies or fungal cultures are, so far, mostly obtained by Sanger sequencing directly applied to PCR products. That is, without a cloning step to isolate different amplicons, if the SSU rRNA gene, ITS and LSU rRNA gene are the amplified regions. (Selosse, Vincenot & Öpik 2016). This is amazing mostly in the case of ITS which represents a vastly polymorphic marker and so an influential tool for taxonomic drives at species level (Nugent & Saville 2004).

The ITS rDNA region reachable with widespread primers can necessarily distinguish between most fungal species and is also the most abundant fungal marker in open databases. The DNA cluster is multicopy marker present in fungal genome in 1-200 copies. Although the multicopy nature of this sequence facilities tracking down ITS amplicons from low quality DNA, it moreover utterly limits its importance for the quantification of the comparative abundance of fungal taxa. The multicopy nature furthermore results in intraspecies and intragenomic changeability. Wide-ranging studies representing the distribution of intragenomic ITS rDNA inconsistency, among fungi, are lacking although its extent is usually reflected to be low. Even additional variability could be unseen in possibly unrecognized rDNA pseudogenes. The ITS data set may supposedly contain deeper intragenomic paralogous which increased number of singletons and OTUs in the ITS data set (Větrovský et al. 2016).

This proposes that three replicates of two different annealing temperatures and a larger number of target reads may be required for the ITS2 primers to obtain a similar number OTUs as new LSU primers (Asemaninejad et al. 2016). To date some 500, 000 fungal ITS sequences have been produced by the technical community and are accessible for reference in the International Nucleotide Sequence Database Collaboration (INSDC), European Nucleotide Archive (ENA) and the DNA Data Bank of Japan (DDBJ). The mycological community deliver ITS sequences of high integrity and helpfulness for the scientific community at large number. Three hundred bases are routinely sufficient to cover at least one end of the much conserved 5.8 S gene in the centre of the ITS region (Nilsson, Abarenkov & Kõljalg 2016).

Many fungal taxonomy revisions have used DNA sequence of the non-coding ITS regions of the nuclear ribosomal RNA repeat for resolving relationships at the genus and species level. In fungal taxonomy research uncertainties in phylogenetic analysis, using the ITS-1 make species level discrimination hard. Resolution of phylogenies in these cases is habitually probable, using the nuclear LSU of rRNA. This region (LSU) is less mutable than the ITS-1 region and is freely amplified from a large group of Basidiomycetes fungi (Nugent & Saville 2004).

However, ongoing examination of the ITS for fungal diversity has discovered that, the ITS region is also variable to align over distantly related taxa, and thus unable to surely place sequences at the level of family, order or class for which no closely matching reference sequences exist. Overall, fungal identification and taxonomic analyses, using ITS remain difficult. Since the LSU primers, provide abundant read counts in environmental samples, have low rates of data loss, and cover a breadth of fungal taxa. It has been advised that LSU provide a promising addition to complement and counterpart other universal primers in metagenomics studies (Asemaninejad et al. 2016).

Fungal research repeatedly uses the nuclear ribosomal ITS, now the most accredited taxonomic barcode for fungi. The ITS and large subunit (LSU) rRNA genes are more common in taxonomic research. The multi-copy structure of rDNA facilitates its amplification by PCR, and makes it very suitable for taxonomic barcoding, but ITS sequences are part of the ribosomal DNA locus that behaves as a single Mendelian locus. ITS-based fungal OTUs are most universally defined at the 97% sequence similarity threshold: although this also accounts for sequencing errors, it acknowledges the existence of ITS polymorphism within most fungal species. There are cases, where the ITS region shows larger within-species variation, compared to between-species distance, than single-copy genes (Harder et al. 2013).

Many studies have investigated species complexes, using crossing tests together with phylogenies based on ITS sequences. However, as can be expected there are exceptions to monophyly of ITS within species (Aanen, Kuyper & Hoekstra 2001). For ITS and Fungi, phylogenetic inference is also complicated by the difficulty to align ITS sequences meaningfully for groups above or even at the genus level, and that it is slower than clustering algorithms based on heuristic pairwise alignments. For fungal ITS, it would also require that we solve the problem of aligning the sequences. In most of these situations, barcoding regions such as the ITS may not be the most appropriate and it may be better to use, for example, the 5' end of the large subunit of ribosomal DNA in a phylogenetic context, or to try to measure the trait or function more directly, for example by enzyme activity or using meta transcriptomic. Genome mining from fungarium specimens improves resolution of the mushroom tree of life. Moncalvo et al.

(2000) provided the first attempt at a large-scale molecular phylogeny of the Agaricales.

The result has been useful for circumscribing some groups at the generic and familial levels and for understanding broad patterns of phylogenetic distribution of various taxa and morphologies. Many fungal taxonomy studies have used DNA sequence of the non-coding internal transcribed spacer (ITS) regions (locus 1 and 2) of the nuclear ribosomal RNA repeat for resolving relationships at the genus and species levels. The region ITS is used by to differentiate illicit fungi of the *Psilocybe* genera and the ITS-based identifications appeared superior to those based on multi-locus analysis. In fungal taxonomy research, ambiguities in phylogenetic analysis, using the ITS make species level discrimination difficult with the idea of confirming (Zent & Zent 2012). In molecular systematics, ITS rDNA sequencing is the most reliable approach for the fungal identification to species level. However, in many forensic laboratories, the profiling of microsatellites is the preferred method of choice and nuclear DNA sequencing is not commonly used, except for some specialized laboratories that carry out mitochondrial analysis whenever the nuclear DNA is degraded. There are cases where two or more taxa that are morphologically and ecologically distinct, cannot be distinguished based on ITS rDNA marker. In addition, sequencing of the ITS region does not allow for individualization of samples, as it is highly conserved within a species (Kallifatidis et al. 2014).

PCR products produce more accurate data by sequencing of the ITS. DNA sequences within the ITS locus, that are conserved within each genus, are used to produce a DNA based test that will identify the presence of hallucinogenic fungi and will further characterise the genus of fungi present. The size of the ITS product is also indicative of the species present. Seized fungal material that cannot be identified by morphological means and where the chemical hallucinogenic compounds have degraded, can be identified by this novel DNA test (Lee, Cole & Linacre 2000a).

The ITS region consists of two non-coding nuclear DNA regions (ITS1, ITS2) that separate three ribosomal RNA genes (rRNA): 18S, 5.8S and 28S. On the terminals of this complex, there are external transcribed spacer (ETS) regions:

ETS1 and ETS2, situated respectively within the 5' and 3' terminal region. ITS fragments are extremely useful in species identification due to the presence of length and sequence polymorphisms. Studies carried out to date have demonstrated that the ITS regions yield excellent results in molecular systematics at the species level, as well as in the determination of interspecies geographical variation. These fragments are present in many copies, so they can be amplified even in markedly degraded material, which is of great significance in studies performed for forensic purposes. The methodology employed in species identification of various poisonous mushrooms, just as in the case of mushrooms from the *Psilocybe* genera, consists mostly in the analysis of sequences in the ITS and nLSU regions. The researchers propose application of the analysis of sequences in the ITS1 and ITS2 regions to determine species of mushrooms from the *Amanita* genus for clinical purposes

(Zuber, Kowalczyk, Sekuła, et al. 2011).

This is surprising particularly in the case of ITS, which represents a highly polymorphic marker and consequently a powerful tool for taxonomic purposes at species level. Fragments encoding ribosomal RNA are separated by noncoding fragments, which are removed after the transcription phase. These so-called internal transcribed spacers (ITS1 and ITS2) and non-transcribed intergenic spacers (IGS) are characterized by the presence of polymorphisms of length and sequence, which make them highly useful for species identification. There are primers serving in the amplification of both ITS regions, including the 5,8S coding region, as well as species-specific primers targeting the ITS showed that hallucinogenic mushrooms from the genus *Psilocybe* can be differentiated on the basis of differences in the lengths of ITS1 PCR products. Studies conducted on the polymorphism of the ITS region, encompassing mushrooms belonging to many taxa, show the usefulness of these regions as a basic universal marker in the fungi kingdom. Other studies show that the high degree of polymorphism of sequences of the ITS region may lead to ambiguous identifications of closely related species (Kowalczyk, M et al. 2015). Molecular identification of fungi usually relies, at least in the first attempts, on sequencing the nuclear ribosomal internal transcribed spacer (ITS) region, the formal fungal barcode. The largest database tailored for fungal ITS sequences is UNITE (Abarenkov et al. 2010)

(http://unite.ut.ee) for fungal ITS sequences and offers extensive capacities for analysis and third-party annotation of sequences to its users (Nilsson et al. 2014). The internal transcribed spacer (ITS) is a popular barcode marker for fungi and in particular the ITS1 has been widely used for the anaerobic fungi (phylum Neocallimastigomycota). A good number of validated reference sequences of isolates as well as a large number of environmental sequences are available in public databases. Its highly variable nature predisposes the ITS1 for low level phylogenetics (Koetschan et al. 2014).

Amplicon sequencing of the ribosomal ITS region and whole genome sequencing of fungal isolates can be used as the most discriminative approach in genetic research of various fungal pathogens like *Aspergillus* and *Candida* species and as a tool in taxonomic identification (Zoll et al. 2016). The ITS region cannot distinguish between all species. Distinguishing between species using the ITS region also has other limitations, such as dependency on read length and incompleteness in public databases (Dannemiller et al. 2014). However, ongoing examination of the ITS for fungal diversity has revealed that the ITS region is too variable to align over distantly related taxa, and therefore unable to confidently place sequences at the level of family, order or class for which no closely matching reference sequences exist.

Recently, a multi-lab consortium investigating the best genetic marker for fungal taxonomy identified the ribosomal internal transcribed spacer (ITS) region as the ideal marker gene. The automated processing of fungal ITS region DNA sequence data should include the following:

(i) Removal of primer sequences, adaptors, and multiplex tags,

(ii) Quality trimming based on sequencing quality scores and length, (iii) High-throughput comparison of retrieved sequences with an ITS sequence databases, and

(iv) The ability to select, taxonomically classify, and quantify the sequence abundance of database search results.

Three replicates of two different annealing temperatures and a larger number of target reads may be required for the ITS2 primers (Asemaninejad et al. 2016).

The nuclear ribosomal internal transcribed spacer (ITS) region is the formal fungal barcode and in most cases the marker of choice for the exploration of fungal diversity in environmental samples. Species represented by at least two ITS sequences in the international nucleotide sequence databases are now given a unique, stable name of the accession number type and their taxonomic and ecological annotations are corrected as far as possible through a distributed, third-party annotation effort. These reference sequences are released (http://unite.ut.ee/repository.php) for use by the scientific community in, for example, local sequence similarity searches and in the QIIME pipeline. The system and the data will be updated automatically as the number of public fungal ITS sequences grow. The nuclear ribosomal internal transcribed spacer (ITS) region has a long history of use as a molecular marker for species-level identification in ecological and taxonomic studies of fungi. It offers several advantages over other species-level markers in terms of high information content and ease of amplification, and it is recently designated the official barcode for fungi UNITE mirrors the fungal ITS sequences in the International Nucleotide Sequence Databases (INSD: GenBank, EMBL (European Molecular Biology Laboratory) and DDBJ.

The ~300, 000 public fungal ITS sequences constitute a poor candidate for the basis of taxonomic annotation of newly generated sequences, especially when used in conjunction with fully automated pipelines. Only about half of these sequences are annotated to the level of species. This half represents approximately 20, 000 different species. From a bioinformatics point of view, an ideal representative sequence should cover the full ITS region and should preferably not feature many IUPAC DNA ambiguity symbols (Cornish-Bowden 1985) or manifest signs of a potentially compromised technical / read quality related nature (Kõljalg et al. 2013).

The nuclear ribosomal large subunit, a popular phylogenetic marker in certain groups, has superior species resolution in some taxonomic groups, such as the early diverging lineages and the Ascomycete yeasts, but is otherwise slightly inferior to the ITS. The nuclear ribosomal small subunit has poor species-level resolution in fungi. ITS will be formally proposed for adoption as the primary fungal barcode marker to the Consortium for the Barcode of Life, with the

possibility that supplementary barcodes may be developed for particular narrowly circumscribed taxonomic groups. Posttranscriptional processes split the cistron and removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are usually referred to as the ITS region. In an expanded dataset of ITS sequences from our fungal DNA barcoding database, the highest variation is most often found in the early diverging lineages. ITS combines the highest resolving power for discriminating closely related species with a high PCR and sequencing success rate across a broad range of fungi. If the taxa is taxa with low ITS interspecific variability, secondary markers must be used to accurately report genetic diversity one or any combination of the six genes (Schoch et al. 2012).

There are cases where the ITS region shows larger within-species variation, compared to between-species distance, than single-copy genes. Further studies are needed to determine how well suited the coalescence model is to model the evolution of the ITS region. The speciation rates, substitution rates and reasonable population sizes and generation times may be different in other organism groups than fungi and other barcoding regions than ITS. However, the general problem is within-species variation of the ITS region in fungi. Many studies have investigated species complexes, using crossing tests together with phylogenies based on ITS sequences between-compatibility group distances vary. There are also many examples where the largest within compatibility group ITS sequence distance is larger than the minimum between-compatibility group distance (Ryberg 2015).

Species represented by at least two ITS sequences in the international nucleotide sequence databases are now given a matchless, stable name of the accession number type and their taxonomic and ecological annotations are modified as far as possible through a distributed, third-party annotation effort. Although the commonly used internal transcribed spacer region of rDNA (ITS) is well suited for taxonomic identification of fungi, the information on the relative abundance of taxa and diversity is negatively affected by the multicopy nature of rDNA and the existence of ITS paralogues. Moreover, due to high variability, ITS sequences cannot be used for phylogenetic analyses of unrelated taxa. Since the first mycological study applying NGS (Öpik et al. 2009), parts of the rDNA cluster

have been exclusively used as a molecular marker. The ITS rDNA region, accessible with universal primers, can sufficiently distinguish between most fungal species and is also the most abundant fungal marker in public databases. The rDNA cluster is a multicopy marker present in fungal genomes in 1–200 copies; although the multicopy nature of this sequence facilitates obtaining ITS amplicons from low-quality DNA, it also seriously limits its value for the quantification of the relative abundance of fungal taxa. The multicopy nature also results in intraspecies and intragenomic variability. Comprehensive studies mapping the distribution of intragenomic ITS rDNA variability among fungi are lacking, although its extent is generally considered to be low with the exception of the Glomeromycota. ITS rDNA is proposed as the universal fungal barcode for its interspecific variability and the availability of conserved primer sites. However, its intragenomic variability could represent a potential limitation for the study of fungi from environmental DNA. The variable length of the ITS region represents another important problem because there is a strong PCR bias against species with longer amplicons, an issue that is found to largely affect the results of community studies. ITS, thus not be able to separate newly diverging species in most cases, and variances in communities based on ITS-MOTUs include processes acting on long time distances. There are cases where the ITS region shows higher within-species deviation, linked to between-species distance, than single-copy genes. Numerous studies have explored species complexes, using crossing tests together with phylogenies based on ITS sequences. However, as can be expected, there are exemptions to monophyly of ITS within species (Ryberg 2015).

DNA sequences within the ITS locus, that are conserved within each genus, are used to produce a DNA based test that classify the existence of hallucinogenic fungi and will further characterise the genus of fungi present. The size of the ITS product is likewise suggestive of the species present. Fungal material that cannot be identified by morphological means and where the chemical hallucinogenic compounds have degraded, can be identified by this novel DNA test (Lee, Cole & Linacre 2000a). Ratio, 50 ng of DNA are used for species identification based on Internal Transcribed Region positioned between the 18S- and the 28S rDNA

genes as formerly well accepted for its capability to classify yeast to the species level (Gambaro et al. 2015).

Many of the species share duplicate or nearly ITS rDNA alleles. Molecular (DNA sequence) data have appeared as critical information for the taxonomic identification of plant pathogenic fungi, with the nuclear ribosomal internal transcribed spacer (ITS) region, being the most popular marker. However, international nucleotide sequence databases are gathering numerous sequences of compromised or low-resolution taxonomic annotations and substandard technical quality, making their use in the molecular identification of plant pathogenic fungi challenging. Molecular identification of fungi typically trusts, at least in the first attempts, on sequencing the nuclear ribosomal internal transcribed spacer (ITS) region, the formal fungal barcode. The largest database tailored for fungal ITS sequences is UNITE (http://unite.ut.ee) (Abarenkov et al. 2010) and offers extensive capacities for analysis and third-party annotation of sequences to its users (Nilsson et al. 2014).

Two highly variable spacers (ITS1 and ITS2) of nuclear ribosomal internal transcribed spacer (ITS) region as a prime choice for molecular identification of fungi are generally species specific, whereas the intercalary 5.8S gene is exceedingly conserved. For sequence clustering and BLAST searches, it is often gainful to rely on either one of the variable spacers but not the conserved 5.8S gene to identify and extract ITS1 and ITS2 from large taxonomic and environmental data arrangements. However, it is often difficult and many ITS sequences are inaccurately delimited in the public sequence databases. The region is composed of two highly variable spacers ITS1 and ITS2 which, conjointly or disjointedly, are often species specific and the intercalary, much conserved 5.8S gene. The sequence conservation in the adjacent genes, tied with numerous copies of the ribosomal operon, makes primer design and PCR amplification of the ITS region straightforward even from low-DNA quantity substrates such as old herbarium specimens and soil. Undeniably, the ITS region is recently designated the formal barcode for fungi for many reasons. The ITS region is nevertheless not a barcoding marker without potential shortcomings. Complications include primer bias, differing evolutionary rates in different fungal

lineages and the presence of several different copies within a single individual. A possibly lesser-known obstacle with the ITS region in the context of molecular identification lies in its composite nature. The ITS1 and ITS2 spacers, on the other hand, are very variable. To subject sequences highlighting both variable and conserved parts to similarity searches such as BLAST in the International Nucleotide Sequence Databases (INSD) does not continuously produce the intended or correct results from the perception of species identification. The conserved sequence parts likely find a match in the databases regardless of whether or not the variable part does and so the outcome of the BLAST search may be more dependent on the length of the conserved element than the information content in the variable one. This would not be a concern, if the reference databases featured an exhaustive taxon sampling of sequences of comparable length. Unluckily, ITS sequence data are available only for 1-5% of the estimated 1-5 million species of fungi and the public fungal ITS sequences come in very different degrees of coverage of the region, notifying against cursory – or fully automated – check of BLAST results. Around 11% of the 86, 000 BLAST searches undertaken produced a different result (nonsynonymous species name) depending on whether the full ITS region or just the variable regions, is used in the search. If the goal is to identify species (or finding other sequences from the same species, with or without a full Latin name), the BLAST search using either ITS1 or ITS2 may be desirable to using the full-length sequence. A multiple ITS sequence alignment inspected in the light of the guidelines is a good way to demarcate ITS1 and ITS2, but such manual approaches become ungainly for larger data sets. To undertake it with data sets produced by high-throughput DNA sequencing techniques such as pyrosequencing – where the number of sequences may exceed hundreds of thousands – is intractable (Bengtsson‐Palme et al. 2013).

A decent number of authenticated reference sequences of isolates as well as a large number of environmental sequences are obtainable in public databases. The very variable nature biases of the ITS1 makes it suitable for low level phylogenetics (Koetschan et al. 2014). The ITS region cannot distinguish between all species. Distinguishing between species using the ITS region also has other limitations, such as dependency on read length and incompleteness in

public databases (Dannemiller et al. 2014). However, satisfactory taxonomic identification remains problematic in the kingdom fungi due to the vast, mostly unexplored diversity and the absence of reliable and richly annotated reference sequences (Kõljalg et al. 2013).

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA from fungi, the formal barcoding region in this kingdom diversity, two 514-base pair long sequences (median length of the fungal ITS region) that separated at the last glacial maximum (19–26.5 kyr. ago). ITS will therefore not be able to separate recently diverging species in most cases, and differences in communities based on ITS-MOTUs include processes acting on long time spans. There are cases where the ITS region shows larger within-species variation, compared to between-species distance, than single-copy genes. The speciation rates, substitution rates, realistic population sizes and generation times may be different in other organism groups than fungi and other barcoding regions than ITS. However, the general problem is within-species variation of the ITS region in fungi. Many studies have inspected species complexes using crossing tests together with phylogenies based on ITS sequences between compatibility group distances vary. There are also many examples, where the largest within compatibility group ITS sequence distance is larger than the minimum between-compatibility group distance. For fungal ITS, it would also require that we resolve the problem of aligning the sequences (Ryberg 2015). Amplicon sequencing of the ribosomal ITS region and entire genome sequencing of fungal isolates can be used as the most discriminative approach in genetic research of various fungal pathogens like Aspergillus and Candida species and as a tool in taxonomic identification (Zoll et al. 2016). The ITS data set may theoretically contain deeper intragenomic paralogues, which increased number of singletons and OTUs a in the ITS data set. While the ITS2 primers recovered as many no target sequences per samples, they yielded far fewer fungal OTUs signifying a much smaller range of higher taxa of fungi (Asemaninejad et al. 2016). The overall possibility of accurate species identification using ITS is comparable with the success reported for the two-marker plant barcode structure. Advanced species identification success can be estimated in the main macro-fungal groups in Basidiomycota and

somewhat minor success can be expected in the economically significant micro-fungal groups in filamentous Ascomycota (Schoch et al. 2012).

In this study we set out to extend the use of ITS sequences to the identification of illicit fungal species. Also, we use a typical phylogenetic analysis of nucleotide sequences from ITS region and define these sequences, do not allow adequate discrimination of species. We select a subset of the species unsolved by the ITS sequence to determine if the nLSU rRNA sequences offer better phylogenetic signal. The resulting nLSU-based trees obviously separate fungal species containing hallucinogens from those that do not (Jeewon & Hyde 2007).

10.6.3 LSU / SSU (Advantages and Disadvantages)

The nuclear ribosomal large subunit, a popular phylogenetic marker in certain groups, has superior species resolution in some taxonomic groups, such as the early deviating lineages and the ascomycete yeasts, but is then marginally inferior to the ITS. The nuclear ribosomal small subunit has poor species-level resolution in fungi. The 18S nuclear ribosomal small subunit rRNA gene (SSU) is commonly used in phylogenetics and although its homolog (16S) is often used as a species diagnostic for bacteria, it has fewer hypervariable domains in fungi. The 28S nuclear ribosomal large subunit rRNA gene (LSU) sometimes discriminates species on its own or combined with ITS. For yeasts, the D1/D2 region of LSU is adopted for characterizing species long before the concept of DNA barcoding is promoted (Schoch et al. 2012).

The 18S rDNA (small subunit; SSU), which evolves rather gradually and is pretty conserved, has been used to provide insights into the phylogeny of far interrelated organisms, mostly at the ordinal and family level. The 28S (large subunit; LSU) is reasonably preserved but offers adequate variant to study relationships at the generic as well as species level (Jeewon & Hyde 2007). All eukaryotic and prokaryotic cells contain rRNA genes which are broadly conserved throughout evolutionary time and they are used for phylogenetic classification. The LSU (28S) region has been used widely for fungal phylogeny and taxonomic placement. This region contains the D1 and D2 hypervariable

domains which are valuable for species identification in various fungal groups (Dizkirici et al. 2018).

Two primer sets pointing the D1 region of the LSU of ribosomal DNA to estimate fungal diversity. These primers can be functional for discovery of key fungal groups to recover a wide variety of fungal taxa with confident upper-level placement and probable species or species group identification. Regrettably no primers have been developed to target the D1 region through a wide diversity of fungi with a right short amplicon for the Illumina MiSeq platform (Asemaninejad et al. 2016). Further studies among mushroom families and genera that trust on nLSU sequences are expected since this locus is effortlessly amplified and phylogenetically valuable (Matheny et al. 2002).

Since LSU provide ample read counts in environmental samples, have low rates of data loss and cover a breadth of fungal taxa, it provides a universal primer in metagenomics projects. The region of LSU between bases 150 and 550 from different fungal groups has been used to query Genbank using BLAST, separately specifying all orders of Agaricomycetes and other Basidiomycota known from soil. Both primers set (LSU) amplify a 300 bp region of the rDNA LSU which delivers reliable placements of sequence clusters within the fungi and together they yield a wide array of fungi from Chytrids mushrooms (Asemaninejad et al. 2016). Probable multi-copy variations in the sequences of the nLSU copies found within an individual, could pose a challenge when it comes to species identification and information on intra-specific variation is still to be determined (Kallifatidis et al. 2014). It is very demanding to classify the original species of the MMs by morphological observation (Maruyama et al.

2003).

The rRNA gene has been investigated for a large subunit (LSU) of several MMs to classify them by a genetic approach. The phylogeny of species of MMs based on the partial sequence has been examined (about 970 bp) of the LSU. The DNA sequence analysis is convenient for the classification of MMs. When an additional region of the nuclear large subunit (nLSU) in ribosomal DNA sequences is employed, which is categorized by a lower degree of polymorphism, it is been able to define the species of those representatives of

hallucinogenic mushrooms, the identification of which has been uncertain in the case of analysing sequence polymorphism of the ITS1 fragment. Pursuant to the achieved results, the analysis of the ITS1 and nLSU regions is proposed as a reliable method. Based on overall performance in species discrimination, SSU has almost no barcode gap and the worst combined PCI, and it can be eliminated as a candidate locus (Parisod & Broennimann 2016).

The methodology employed in species identification of various hallucinogenic mushrooms, consists typically in the analysis of sequences in the ITS and nLSU regions (Zuber, Kowalczyk, Sekuła, et al. 2011). Resolution of phylogenies is often possible using the nuclear large subunit of rRNA (nLSU, 28S). Using all variable characters in the ITS sequence is a phylogenetic approach for fungal identification to the level of species. The resulting nLSU-based trees clearly separate fungal species containing hallucinogens from those that do not. It is proposed that a sequence database of the ITS and the first 900 bp of the divergent nLSU region would provide a reliable and automated approach to the identification of hallucinogen containing fungal isolates. The data shows that combining analyses of the ITS and nLSU sequence variation has the resolving power to distinguish very closely species and separated hallucinogenic and non-hallucinogenic *Psilocybe* species (Nugent & Saville 2004).

The eukaryotic rRNA cistron consists of the 18S, 5.8S and 28S rRNA genes transcribed as a unit by RNA polymerase I. Posttranscriptional procedures split the cistron, removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are typically referred to as the ITS region. The 18S nuclear ribosomal small subunit rRNA gene (SSU) is normally used in phylogenetics and while its homolog (16S) is regularly used as a species diagnostic for bacteria, it has less hypervariable domains in fungi. The 28S nuclear ribosomal large subunit rRNA gene (LSU) occasionally discriminates species on its own or combined with ITS. For yeasts, the D1 / D2 region of LSU is adopted for characterizing species long before the concept of DNA barcoding is promoted. LSU and SSU performs poorly, each missing a significant barcode gap. Overall, ribosomal markers have fewer problems with PCR amplification than protein-coding markers. Based on overall performance in species discrimination, SSU has almost no barcode gap, the worst combined PCI and it

can be excluded as a candidate locus. LSU, a preferred phylogenetic marker among many mycologists, has virtually no amplification, sequencing, alignment or editing problems and the barcode gap is superior to the SSU. However, across the fungal kingdom, ITS is generally superior to LSU in species discrimination and has a more clearly definite barcode gap. Total ribosomal markers have fewer problems with PCR amplification than protein coding markers. Based on whole performance in species discrimination, SSU has nearly no barcode gap and the poorest combined PCI and it can be eliminated as a candidate locus (Schoch et al. 2012).

The large subunit of the nuclear ribosomal RNA (LSU), a favoured phylogenetic marker among many mycologists, has practically no amplification, sequencing, alignment or editing problems and the barcode gap is superior to the Small Subunit of rRNA (SSU). However, across the fungal kingdom, ITS is usually superior to LSU in species discrimination and has a more obviously definite barcode gap (Purty & Chatterjee 2016). Subsequently LSU primers offer plenty read counts in environmental samples and have low rates of data loss and cover an extent of fungal taxa. We advise that they provide an encouraging count to complement and equal other common primers in metagenomics studies (Asemaninejad et al. 2016). Metagenomics which can be used for sequencing of novel species from environmental specimens and can likewise be used for species identification of bacteria and fungi by targeted sequencing of the 16s and ITS regions of the rRNA genes correspondingly (Ahmed 2016). The use of ITS of the rDNA region and the nLSU sequence data for the identification of mushroom species is also proposed. However, possible multicopy variants in the sequences of the nLSU copies found within an individual could pose a challenge when it comes to species identification and information on intra-specific variation is still to be determined (Kallifatidis et al. 2014). The neighbouring SSU (immediately upstream of ITS1) and LSU (immediately downstream of ITS2) genes are much conserved, as is the intercalary 5.8S gene. Distinguishing the individual components of the ITS region is not trivial however. Whereas SSU, 5.8S and LSU are conserved, they are regularly too variable for simple pattern matching approaches via regular expressions for their identification (Bengtsson - Palme et al. 2013).

The D1 variable region of the large ribosomal subunit is an attractive alternate, since it (often together with D2) has proven useful in species-level identification and phylogenetic reconstruction in various fungal groups at a comparatively low cost. Regrettably, no primers have been developed to target the D1 region across a wide diversity of fungi with a short amplicon appropriate for the Illumina MiSeq platform. The region of LSU between bases 150 and 550 from diverse fungal groups is used to query GenBank using BLAST, distinctly specifying all orders of Agaricomycetes and other Basidiomycota known from soil. Together, the LSU primers recovered 127 genera and 28 species that are not obtained using the ITS2 primers, but the ITS2 primers recovered 10 unique genera and 16 species that are not obtained using either of the LSU primers. There is broad agreement at higher taxonomic levels between the fungal communities detected using the LSU primers and ITS2 primers. Although the ITS2 primers recovered as many on-target sequences per sample, they yield far fewer fungal OTUs, representing a much smaller range of higher taxa of fungi (Asemaninejad et al. 2016). A series of manoeuvres such as extraction of genomic DNA, PCR and nucleotide sequencing is very difficult and time consuming (Maruyama et al. 2006). PCR is performed to amplify the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, the internal transcribed spacer 2 (ITS2), and a partial sequence (600 bp) of the large subunit (LSU) of the rRNA gene, including the D1–D2 domains. The primer combination in two fragments of 300 bp each, from obtained genomic DNA is used to amplify the partial sequence of LSU. Furthermore, two primers specific to *Psilocybe* are designed, using the software Geneious and used to amplify the partial LSU in two fragments of 300 bp each. This study created many new sequences which could be the source of numerous investigation (Ramírez-Cruz et al. 2013).

10.6.4 RPB (Advantages and Disadvantages)

RNA polymerase II is the enzyme responsible for transcription of protein-coding genes into pre-mRNA records. RNA polymerase II largest subunit (RPB1) offer appropriate alternatives to other markers to recreate lower-level phylogeny among mushroom-forming fungi (Agaricales, Basidiomycota). Results showed that several gene sections are accessible to these primers yielding a single PCR-

product. Among these gene sections, a novel high fidelity primer pair for RPB already widely used as a phylogenetic marker in mycology, has potential as a supplementary DNA barcode with superior resolution to ITS. The largest subunit of this protein, RPB1, is also of notice, paradoxically, since it possesses the dynamic binding site for a-Amanitin, a toxin formed by several agaric genera such as *Amanita*. Interestingly, the RPB2 but not ITS analysis has showed a high level of significance for differences in the species richness and diversity estimates between the soil fungi. In soil, RPB2 exhibited wide taxonomic coverage of the entire fungal tree of life including basal fungal lineages. The OUT richness and diversity of the entire data set are higher for the ITS data set than the RPB2 data set. A lower effect of nucleotide similarity on the resulting OTU numbers for RPB2 than the ITS data set revealed the RPB2 data set showed only a minor increase. Minor effect of nucleotide likeness on the resulting OUT records for RPB2 than the ITS data set, the RPB2 data set showed simply a slight surge (Větrovský et al. 2016).

The proportions of OTU count and read counts of major fungal groups are close to the reality, when RPB2 is used as a molecular marker, while they are strongly biased towards the Basidiomycota, when using the ITS primers ITS1 / ITS4. Even though the protein coding gene regions often had a higher part of exact identification related with ribosomal markers, low PCR amplification and sequencing success excluded them as candidates for a universal fungi barcode. Among protein coding genes the chief subunit of RNA polymerase II (RPB1) may have potential as a fungal barcode. It is ubiquitous and solitary copy and it has slow rate of sequence variance. Its phylogenetic use is revealed in studies of Basidiomycota, Zygomycota and some Protists. RPB1 primers are developed for the Assembling the Fungal Tree of Life (AFToL) scheme and the locus is included in the consequent. However, its use as a barcode remains unapproved (Schoch et al. 2012).

The low conservancy of the ITS region precludes its use in phylogenetic studies on higher taxonomic ranks. The protein-encoding genes are suitable for this, as the translated amino acid sequences can be utilized for high-quality alignments of unrelated fungi. This could be exceptionally valuable for the proper placement of unknown higher taxonomic level lineages, which are often encountered in

environmental samples for the above reasons, the use of single copy protein encoding genes as alternative markers could be a solution to the multiple problems associated with rDNA-based markers. Although it is recognized that protein-encoding markers, including the RPB2 gene, have a better species-resolving power than rDNA markers, the absence of universal primers is believed to limit their use as potential universal barcodes. There are no commonly used universal primers for RPB2 (Větrovský et al. 2016). The fungal taxon spectra obtained with rbp2 region and ITS1 corresponded, but sequence abundance differs widely, especially in the basal lineages. Although the taxonomic placement of rbp2 sequences is currently more difficult than that of the ITS sequences, its discriminative power, quantitative representation of community composition and suitability for phylogenetic analyses represent significant advantages. The intraspecies inconsistency of the RPB2 gene have to be alike to ITS. The ITS marker appeared to be much more sensitive to chimera formation than RPB2. The selected RPB2 marker region only hardly encloses introns. The RPB2 gene has taxonomic sensitivity superior to the ITS. Even though the exact taxonomic placement of RPB2 sequences is presently more challenging that it is for ITS due to the lower demonstration in GenBank, the phylogenetic discriminative control well calculable representation of the community composition and appropriateness for phylogenetic analysis may represent relative benefits for RPB2 over the usage of ITS and make this molecular marker beneficial for studies in fungal ecology and diversity. The part of solitary copy gene encrypting the second largest subunit of RNA polymerase II (RPB2) is therefore compared with first spacer of ITS as an alternate marker for the analysis of fungal societies.

In addition, the continuous length of the RPB2 amplicon avoids the PCR bias observed in the case of ITS, where length varies mainly among taxa. (RPB2) as an alternative marker for the analysis of fungal communities in soil fungi and their applicability is tested on a comprehensive mock community. In soil, RPB2 exhibited broad taxonomic coverage of the entire fungal tree of life, including basal fungal lineages. Unexpectedly the RPB2 gene produces a more taxonomically diverse set of fungal sequences than the universal ITS primers. The RPB2 data set contained much more nonfungal sequences than the ITS data set. In ITS and RPB2 data sets from environmental samples, a

proportionally similar number of Basidiomycete reads is found in both data sets. The most notable difference between the ITS and RPB2 data sets is the high diversity and abundance of sequences classified into various groups of the basal fungal lineages in the RPB2 data set. The ITS marker seems to be much more sensitive to chimera formation than RPB2. It should be also noted that the distribution of sequences among taxa of the mock community is slightly more even. The single-copy, protein- encoding gene RPB2 may be a viable option for fungal Metabarcoding. Furthermore, the constant length of the RPB2 amplicon avoids the PCR bias observed in the case of ITS, where length varies largely among taxa. The RPB2 gene has taxonomic sensitivity superior to the ITS.

The gene exhibited sufficient variant for the use in phylogenetic analyses and taxonomic assignments, while it amplifies also paralogues. Although the taxonomic placement of rbp2 sequences is presently more difficult than that of the ITS sequences, its discriminative power, quantitative demonstration of community composition and suitability for phylogenetic analyses signify significant advantages. Although it is recognized that protein-encoding markers, including the RPB2 gene, have an improved species-resolving power than rDNA markers, the absence of universal primers is assumed to limit their use as potential universal barcodes. Unpredictably, the RPB2 gene produced a more taxonomically diverse set of fungal sequences than the universal ITS primers.

The RPB2 data set contained much more nonfungal sequences than the ITS data set. In ITS and RPB2 data sets from environmental samples, a proportionately similar number of Basidiomycete reads is found in both data sets. The most remarkable difference between the ITS and RPB2 data sets is the high variety and abundance of sequences categorized into various groups of the basal fungal lineages in the RPB2 data set (Větrovský et al. 2016). Multi-locus markers such as ITS beside with RPB have been presumed to be more fruitful in species identification (Purty & Chatterjee 2016). The single-copy, protein- encoding gene RPB2 may be a feasible choice for fungal Metabarcoding. Some studies show overall agreement in the identity of the fungal genera and fungal classes recovered using this marker and ITS furthermore, the constant length of the RPB2 amplicon avoids the PCR bias observed in the case of ITS, where length varies chiefly among taxa. The RPB2 gene has taxonomic sensitivity superior to

the ITS. While the precise taxonomic placement of rbp2 sequences is presently more problematic than it is for ITS, due to the lesser demonstration in GenBank, the phylogenetic discriminative power, better quantitative representation of the community composition and suitability for phylogenetic analyses may represent relative advantages for rbp2 over the use of ITS and make this molecular marker beneficial for studies in fungal ecology and diversity (Větrovský et al. 2016). The overall possibility of precise species identification using ITS is comparable with the success reported for the RPB, ITS performed as a close second to RPB1 (Schoch et al. 2012).

10.6.5 EF (Advantages and Disadvantages)

The partial elongation factor EF1α sequence is amplified using the forward primer EF-595F and reverse primer EF-1160R. The EF1α sequence included two partial and one whole exon and two introns. The elongation factor (EF) complex subunits, however, is acquired such moonlighting functions without alternative forms. One issue about EF is that, it can be changed in composition as the complexity of eukaryotic organisms increase. Although different alternative functions of the EF have been widely reported, recent studies are shedding light on additional functions of the EF. A thorough understanding of these alternate functions of EF is essential for appreciating its biological relevance (Feau et al. 2011).

10.6.6 ISSR (Advantages and Disadvantages)

Universal microsatellite DNA primer (GACA) 4 is a solo oligonucleotide complementary to single repetitive sequences present in the target DNA. This repetitive sequence is called microsatellites (El-Hariri et al. 2010). SSRs are non-coding repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repetitive tandem, which are common in both eukaryotic and prokaryotic genomes. For that reason, many authors recommended the use of microsatellite

as genetic markers, to identify individual genetic inconsistency among closely linked humans, animals and among plants and fungi. Based on present understandings, it is envisioned that the use of ISSR (Inter Simple Sequence Repeat) is the most effective molecular analysis tool for differentiation of species as other methods are not as refined nor easily applied (Tsujikawa et al. 2003).

Inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers are the multilocus marker that in addition to these features does not have the restrictions of other markers such as low reproducibility in RAPD, high costs and complication in AFLP. ISSR being able to create polymorphism outlines among near-organisms and their reproducibility caused to be known as informative markers with a wide range of applications including the study of genetic diversity (Bagherabadi, Zafari & Soleimani 2015). ISSR marker with the whole-genome coverage, accuracy and reproducibility as well as robustness has ascertained to be a promising marker for genetic diversity analysis of numerous plants. Microsatellite analysis is the common approach to study the genetic diversity between fungal isolates. Overall, it can be concluded that ISSR markers are convenient tools to assess genetic variations among closely related strains of *A. bisporus* (Malekzadeh, Shahri & Mohsenifard 2011).

Overall, fungal identification and taxonomic analyses, using ITS remain problematic. The D1 variable region of the large ribosomal subunit is an attractive alternative, since it (often together with D2) has proven useful in species-level identification and phylogenetic reconstruction in various fungal groups at a relatively low cost. Among NGS methods, Illumina MiSeq sequencing is the most effective and extensively used technology globally due to its low rate of error and the lowest cost per million bases, but requires short diagnostic regions of 300 base pairs to be effective. Unfortunately, no primers have been developed to target the ITS, LSU and EF regions across a wide diversity of *Psilocybe* with a short amplicon suitable for the Illumina MiSeq platform. Here we introduce and evaluate three primer sets targeting these three regions to evaluate fungal diversity. We demonstrate how these primers can be applied for discovery of major *Psilocybe* groups to recover a broad range of this taxa with confident higher-level placement (family, order, etc.) and potential species or species-group identification. Internal transcribed spacer (ITS) region primers

The ITS region is now perhaps, the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genomic regions of rDNA (SSU and LSU), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1+ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Gardes & Bruns 1993) (Diagram 10.18.).

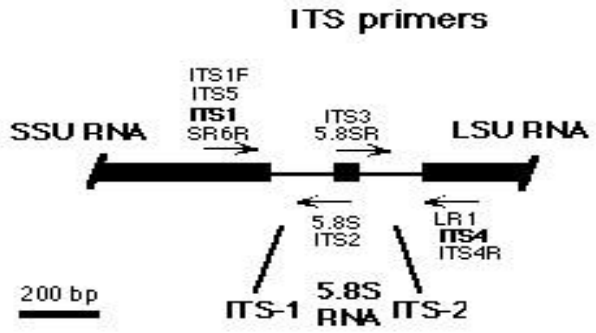


Diagram 10.18 Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA (Gräser et al. 1996)

A useful list of conserved primer sequences for amplification and sequencing of nuclear rDNA from most major groups of fungi, primarily Eumycota, as well as other eukaryotes. Together, these primers span most of the nuclear rDNA coding region, permitting amplification of any desired region. Standard symbols are used for the three primary nucleotides. Primers ending with "R" represent the coding strand (same as RNA). All other primers are complementary to the coding strand. The nuclear-encoded ribosomal RNA genes (rDNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each) arranged in a head-to-toe manner. Each repeat unit has coding regions for one major transcript (containing the primary rRNAs for a single ribosome), punctuated by one or more intergenic spacer (IGS) regions (Diagram 20). In some groups (mostly Basidiomycetes and some Ascomycetous Yeasts), each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vary among groups (Table 10.20.). Several restriction sites are conserved in the rDNA of fungi.

Table 10.20 Small subunit RNA (SR) primers for *Saccharomyces cerevisiae* (Index Fungorum http:// www.indexfungorum.org/)

Primer name	**Sequence (5'-->3')**	**Position within *S. cereviseae* 17S RNA**	**comments**
BMB-'A'	GRATTACCGCGGCWGCTG	580-558	
BMB-'B'	CCGTCAATTCVTTTPAGTTT	1146-1127	
BMB-'C'	ACGGGCGGTGTGTPC	1638-1624	
BMB-BR	CTTAAAGGAATTGACGGAA	1130-1148	
BMB-CR	GTACACACCGCCCGTCG	1624-1640	
SR1R	TACCTGGTTGATQCTGCCAGT	1-21	
SR1	ATTACCGCGGCTGCT	578-564	
SR2	CGGCCATGCACCACC	1277-1263	
SR3	GAAAGTTGATAGGGCT	318-302	
SR4	AAACCAACAAAATAGAA	838-820	
SR5	GTGCCCTTCCGTCAATT	1146-1130	
SR6	TGTTACGACTTTTACTT	1760-1744	
SR6R	AAGWAAAAGTCGTAACAAGG	1744-1763	
SR7	GTTCAACTACGAGCTTTTTAA	617-637	
SR7R	AGTTAAAAAGCTCGTAGTTG	637-617	
SR8R	GAACCAGGACTTTTACCTT	732-749	
SR9R	QAGAGGTGAAATTCT	896-910	
SR10R	TTTGACTCAACACGGG	1181-1196	
NS1	GTAGTCATATGCTTGTCTC		
NS2	GGCTGCTGGCACCAGACTTGC		
NS3	GCAAGTCTGGTGCCAGCAGCC		
NS	CTTCCGTCAATTCCTTTAAG	(similar to BMB-B)	
NS5	AACTTAAAGGAATTGACGGAAG	(is similar to BMB-BR)	
NS6	GCATCACAGACCTGTTATTGCCTC		
NS7	GAGGCAATAACAGGTCTGTGATGC		
NS8	TCCGCAGGTTCACCTACGGA		

BMB = "universal" SSU primers developed by Lane et al., 1985
SR = primers developed by Vilgalys lab
NS = primers described by White et al., 1990

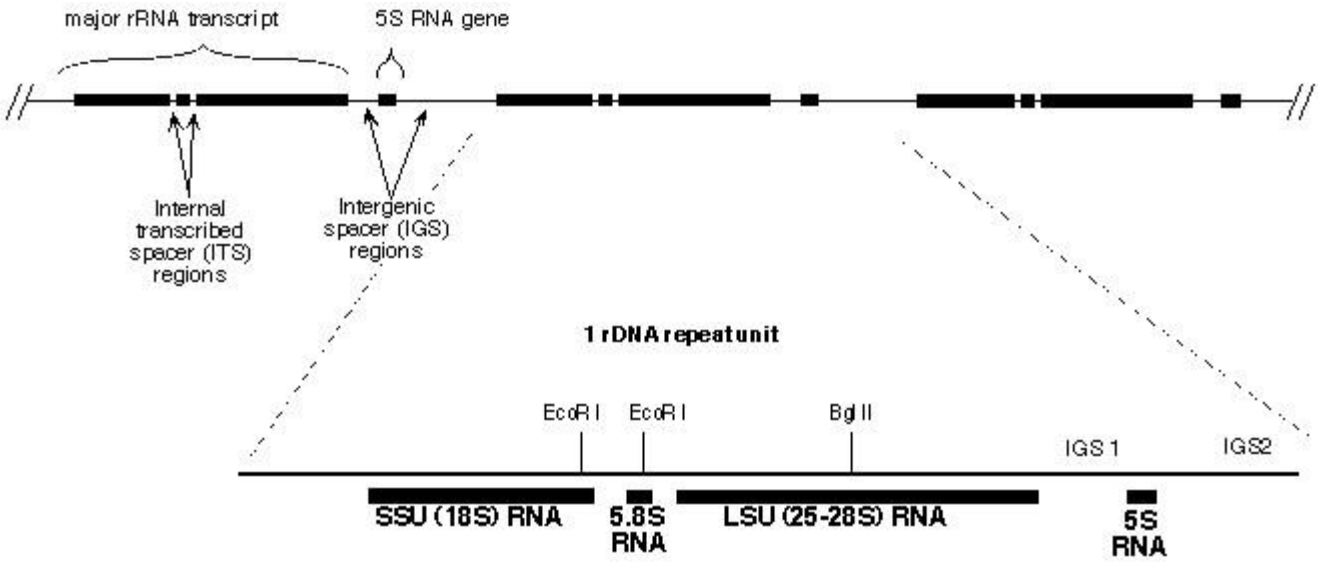


Diagram 10.19 SSU and LSU regions in rDNA
(Index Fungorum (http:// www.indexfungorum.org/)

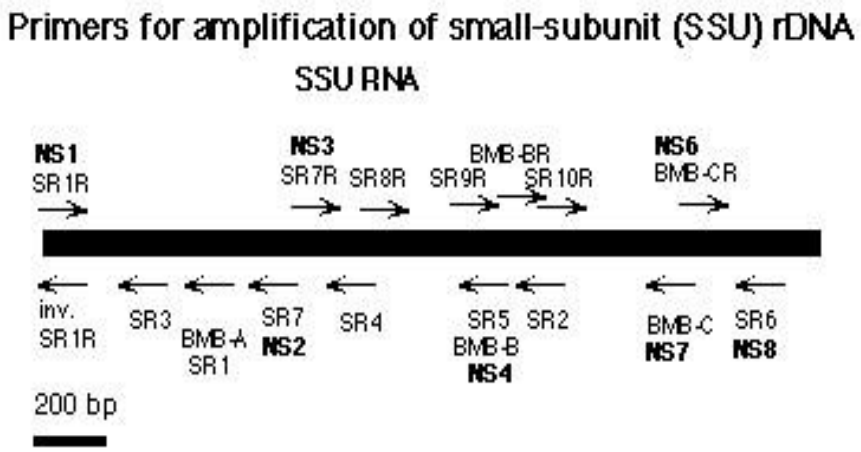


Diagram 10.20 Primers for amplification of (SSU) rDNA
(Index Fungorum http:// www.indexfungorum.org/)

Large subunit RNA (25-28S) primer sequences;

Most molecular systematics studies only utilize the first 600-900 bases from the LSU gene, which includes three divergent domains (D1, D2, D3) that are among the most variable regions within the entire gene (much of the LSU is invariant even across widely divergent taxa). Most of the data in our Agaricales LSU database consists of the first 900 bases from the LSU gene (Diagrams 10.19., 10.20., 10.21.) (Table 10.21.)

Table 10.21 Large subunit RNA (LR) primers for *Saccharomyces cerevisiae* (Index Fungorum http:// www.indexfungorum.org/)

Primer name	**Sequence (5'-->3')**	**Position within *S. cereviseae* rRNA**	**comments**
5.8S	CGCTGCGTTCTTCATCG	51-35 (5.8S RNA)	contains Eco-RI site
5.8SR	TCGATGAAGAACGCAGCG	34-51 (5.8S RNA)	contains Eco-RI site
LR0R	ACCCGCTGAACTTAAGC	26-42	
LR1	GGTTGGTTTCTTTTCCT	73-57	
LR2	TTTTCAAAGTTCTTTTC	385-370	
LR2R	AAGAACTTTGAAAAGAG	374-389	
LR3	CCGTGTTTCAAGACGGG	651-635	
LR3R	GTCTTGAAACACGGACC	638-654	
LR5	TCCTGAGGGAAACTTCG	964-948	
LR6	CGCCAGTTCTGCTTACC	1141-1125	
LR7	TACTACCACCAAGATCT	1448-1432	contains BglII site
LR7R	GCAGATCTTGGTGGTAG	1430-1446	contains BglII site
LR8	CACCTTGGAGACCTGCT	1861-1845	
LR8R	AGCAGGTCTCCAAGGTG	1845-1861	
LR9	AGAGCACTGGGCAGAAA	2204-2188	
LR10	AGTCAAGCTCAACAGGG	2420-2404	
LR10R	GACCCTGTTGAGCTTGA	2402-2418	
LR11	GCCAGTTATCCCTGTGGTAA	2821-2802	
LR12	GACTTAGAGGCGTTCAG	3124-3106	
LR12R	CTGAACGCCTCTAAGTCAGAA	3106-3126	
LR14	AGCCAAACTCCCCACCTG	2616-2599	
LR15	TAAATTACAACTCGGAC	154-138	
LR16	TTCCACCCAAACACTCG	1081-1065	
LR17R	TAACCTATTCTCAAACTT	1033-1050	
LR20R	GTGAGACAGGTTAGTTTTACCCT	2959-2982	
LR21	ACTTCAAGCGTTTCCCTTT	424-393	
LR22	CCTCACGGTACTTGTTCGCT	364-344	

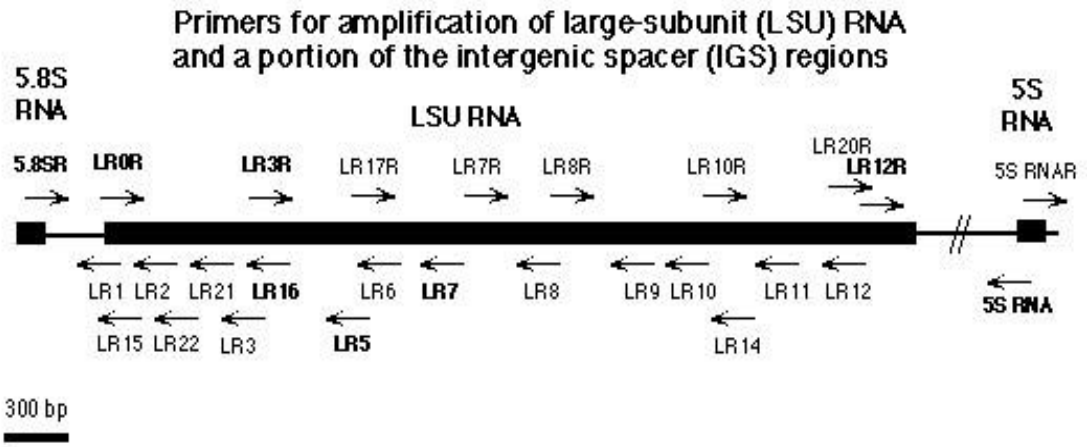


Diagram 10.21 Primers for amplification of (LSU) RNA and IGS
(Index Fungorum (http:// www.indexfungorum.org/)

- Intergenic spacer (IGS) primers (including 5S RNA primer sequences for Basidiomycete fungi);

The greatest amount sequence variation in rDNA exists within the IGS region (sometimes also known as the non-transcribed spacer or NTS region). The size of the IGS region may vary from 2 kb upwards. It is not unusual to find hypervariability for this region (necessitating cloning of individual repeat haplotypes).

Several patterns of organization can be found in different groups of fungi:

1. Most filamentous Ascomycetes have a single uninterrupted IGS region (between the end of the LSU and start of the next SSU sequence), which may vary in length from 2-5 kb or more. Amplification of the entire IGS region requires using primers anchored in the 3' end of the LSU gene (e.g., LR12R) and 5' end of the SSU RNA gene (e.g., invSR1R).

2. In many Ascomycetous Yeasts and nearly all Basidiomycetes, the IGS also contains a single coding region for the 5S RNA gene, which divides the IGS into two smaller regions that may be more easily amplified. Depending on the orientation and position of the 5S RNA gene, the PCR may be used to sequentially amplify either apportion of the intergenic spacer region (IGS) beyond the large subunit RNA coding region (Diagram 10.22.).

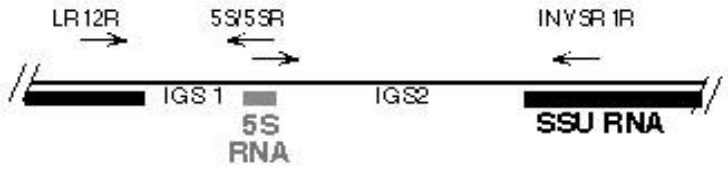


Diagram 10.22 Intergenic Spacer; IGS1 and IGS2 regions Index
(Fungorum http:// www.indexfungorum.org/)

Some examples of the used primers in fungi speciation;

Table 10.22 Some examined primers for amplifying whole ITS
(Fungorum http:// www.indexfungorum.org/)

(ITS3_KYO2/ITS4_KYO3 primers, which amplify the internal transcribed spacer 2 (ITS2) of Ascomycota and Basidiomycota)

Category	Primer name	Position	Primer sequence, 5′–3′	Primer source study
SSU (forward)	NSA3	1514–1535	AAACTCTGTCGTGCTGGGGATA	Martin & Rygiewicz (2005)
	ITS9mun	1618–1635	TGTACACACCGCCCGTCG	Egger (1995)
	NSI1	1644–1663	GATTGAATGGCTTAGTGAGG	Martin & Rygiewicz (2005)
	ITS1-F	1723–1744	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
	ITS1-F_KYO1	1723–1744	CTHGGTCATTTAGAGGAASTAA	This study
	ITS1-F_KYO2	1733–1753	TAGAGGAAGTAAAAGTCGTAA	This study
	ITS5	1737–1758	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
	ITS1	1761–1779	TCCGTAGGTGAACCTGCGG	White et al. (1990)
5.8S (reverse)	ITS2	2024–2043	GCTGCGTTCTTCATCGATGC	White et al. (1990)
	58A2R	2026–2042	CTGCGTTCTTCATCGAT	Martin & Rygiewicz (2005)
	ITS10mun	2026–2043	GCTGCGTTCTTCATCGAT	Egger (1995)
	ITS2_KYO1	2026–2042	CTRYGTTCTTCATCGDT	This study
	ITS2_KYO2	2029–2046	TTYRCTRCGTTCTTCATC	This study
5.8S (forward)	ITS3	2024–2043	GCATCGATGAAGAACGCAGC	White et al. (1990)
	58A1F	2024–2040	GCATCGATGAAGAACGC	Martin & Rygiewicz (2005)
	58A2F	2026–2042	ATCGATGAAGAACGCAG	Martin & Rygiewicz (2005)
	ITS3_KYO1	2026–2043	AHCGATGAAGAACRYAG	This study
	ITS3_KYO2	2029–2046	GATGAAGAACGYAGYRAA	This study
LSU (reverse)	ITS4	2390–2409	TCCTCCGCTTATTGATATGC	White et al. (1990)
	ITS4_KYO1	2390–2409	TCCTCCGCTTWTTGWTWTGC	This study
	ITS4_KYO2	2401–2418	RBTTTCTTTTCCTCCGCT	This study
	ITS8mun	2433–2450	CTTCACTCGCCGTTACTA	Egger (1995)
	ITS4_KYO3	2442–2459	CTBTTVCCKCTTCACTCG	This study
	ITS4-B	2526–2548	CAGGAGACTTGTACACGGTCCAG	Gardes & Bruns (1993)
	NLB4	2558–2577	GGATTCTCACCCTCTATGAC	Martin & Rygiewicz (2005)
	NLB3	2559–2577	GGATTCTCACCCTCTATGA	Martin & Rygiewicz (2005)
	NLC2	2628–2649	GAGCTGCATTCCCAAACAACTC	Martin & Rygiewicz (2005)
	NL6Amun	2767–2786	CAAGTGCTTCCCTTTCAACA	Egger (1995)
	NL6Bmun	2767–2786	CAAGCGTTTCCCTTTCAACA	Egger (1995)
Subset construction only	NS7	1403–1426	GAGGCAATAACAGGTCTGTGATGC	White et al. (1990)
	LR3	3029–3045	CCGTGTTTCAAGACGGG	Vilgalys & Gonzalez (1990)

Both primers whose coverage of fungal taxa was evaluated and those used only for subset construction are shown, together with their position in a reference ribosomal RNA sequence of *Serpula himantioides* (AM946630).
doi:10.1371/journal.pone.0040863.t001

- Conserved domains A to C of RPB1 are amplified with the primer pair gRPB1-A/ fRPB1-C (Fungorum http:// www.indexfungorum.org/):

RPB1-Af, RPB1-Ac	5'-GARTGYCCDGGDCAYTTYGG-3'	forward
RPB1-Cr	5'-CCNGCDATNTCRTTRTCCATRTA-3'	reverse

- Three primers (Ps-int2F 5-GGCWGAACGAGSAGTGCG-3, Ps-Ex2R 5-GCGTAYTCTTCCGAGAGACC- 3 and Ps-Ex3R 5-GCATRACAGTAAGAATCATCC-3) are designed to amplify RPB1 in *Deconica* and *Psilocybe* (Fungorum http:// www.indexfungorum.org/).

- Following three primers for amplifying the RPB2 (Fungorum http:// www.indexfungorum.org/):

RPB2-6F	TGGGGKWTGGTYTGYCCTGC
BRPB2-6F	TGGGGYATGGTNTGYCCYGC
RPB2-7R	CCCATWGCYTGCTTMCCCAT

- Both primer sets (LSU200-F/LSU481-R and LSU200A-F/LSU476A-R) amplify a ~300 bp region of the rDNA LSU, which provides reliable placements of sequence clusters within the fungi and, together they yield a broad array of fungi from Chytrids to mushrooms (Table 10.23.)

Table 10.23 Examined primers for amplifying LSU region in some fungi (Ascomycota) (Fungorum http:// www.indexfungorum.org/)

Name	Position	Sequence (5' to 3')	Nomenclature	Tm	Target Taxa
LSU200-F	200–218	AACKGCGAGTGAAGMGGGA	nu-LSU-200-5^{0}	64	Fungi minus
LSU481-R	462–481	TCTTTCCCTCACGGTACTTG	nu-LSU-481-3^{0}	59	Fungi minus
LSU200A-F[a]	200–218	AACKGCGAGTGAAGCRGYA	nu-LSU-200-5^{0}	63	Ascomycota
LSU476A-R[b]	457–476	CSATCACTSTACTTGTKCGC	nu-LSU-476-3^{0}	59	Ascomycota

- Two primers (LPs1 5-ATGCAGCTCAAAATGGGTGGTAAA-3 and LPs1R 5-CTTTCATTACGCGCTCGGGTTTTC-3) specific to *Psilocybe* are designed. LR0R/LR21 and LPs1/LPs1R are used to amplify the partial LSU in two fragments of 300 bp each (Diagram 10.23.) (Table 10.24.).

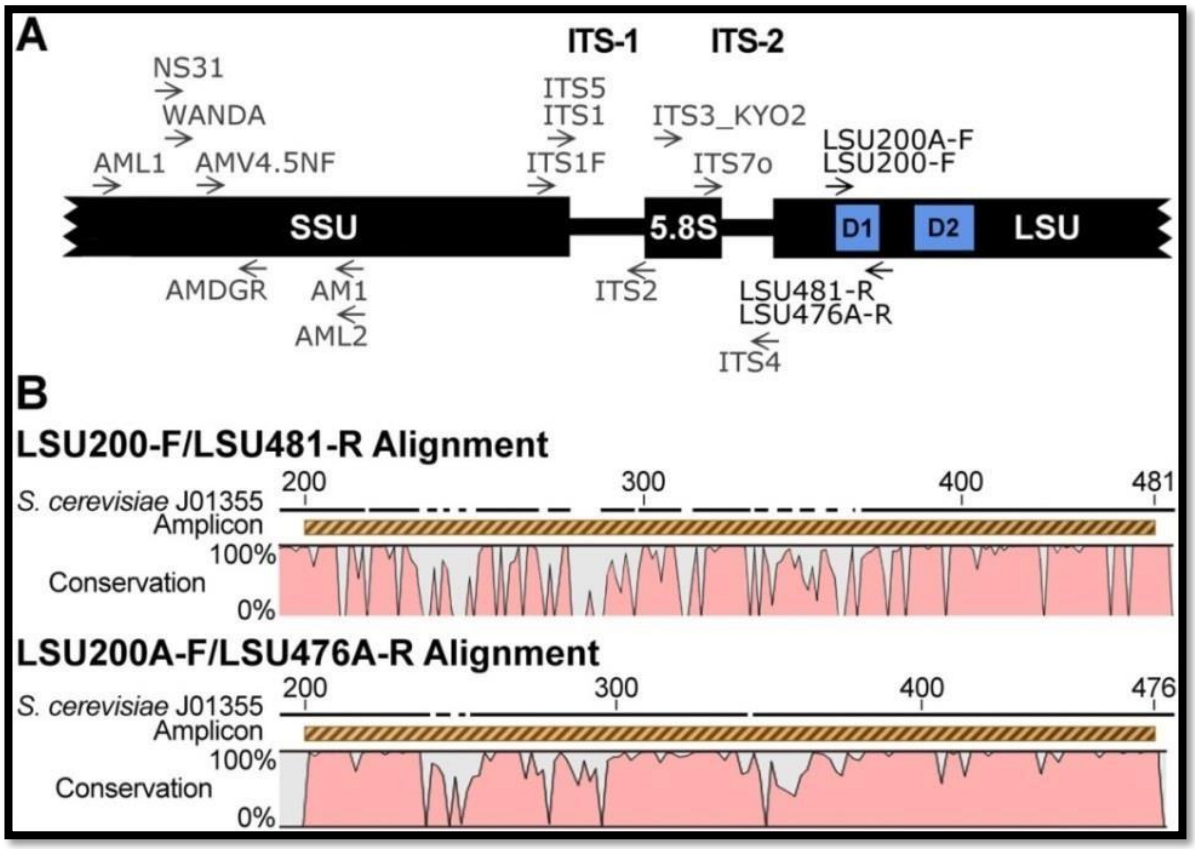


Diagram 10.23 D1 and D2 domain of LSU region and correspondent primers (Fungorum http:// www.indexfungorum.org/)

Table 10.24 Oligonucleotide primer sequences used to amplify fungal rRNA (*Saccharomyces cerevisiae*) (Fungorum http:// www.indexfungorum.org/)

Name	Nucleotide sequence, 5′→3′	Position on *S. cerevisiae* rRNA
SR1-R	TACCTGGTTGATTCTGC	1–17, 17S RNA
5.8S	CGCTGCGTTCTTCATCG	51–34, 5.8S RNA
5.8S-R	TCGATGAAGAACGCAGC	34–51, 5.8S RNA
LR1	GGTTGGTTTCTTTTCCT	73–56, 25S RNA
LR3	GGTCCGTGTTTCAAGAC	654–638, 25S RNA
LR5	ATCCTGAGGGAAACTTC	968–952, 25S RNA
LR6	CGCCAGTTCTGCTTACC	1141–1125, 25S RNA
LR7	TACTACCACCAAGATCT	1448–1422, 24S RNA
LR7-R	AGATCTTGGTGGTAGTA	1422–1448, 25S RNA
LR12	GACTTAGAGGCGTTCAG	3126–3110, 25S RNA

- RPB1 sequencing primers included gRPB1-A forward, fRPB1-C reverse and an additional primer, aRPB1-B reverse, designed from sequence comparisons of *Inocybe* species. RPB1 sequencing primers specific to *Inocybe* included B forward (59-TTGTCCCTTGGCACCCGAG-39) and B.5 reverse (59-CCGCTTAGTTTCCTCATTATCG-39). Primers used to amplify the 59 end of the nLSU gene included 5.8SR and LR7, along with sequencing primers LR0R, LR3R, LR16, and LR5, are obtained from (http://www.botany.duke.edu/fungi/mycolab/primers.htm) (Diagrams 10.24., 10.25).

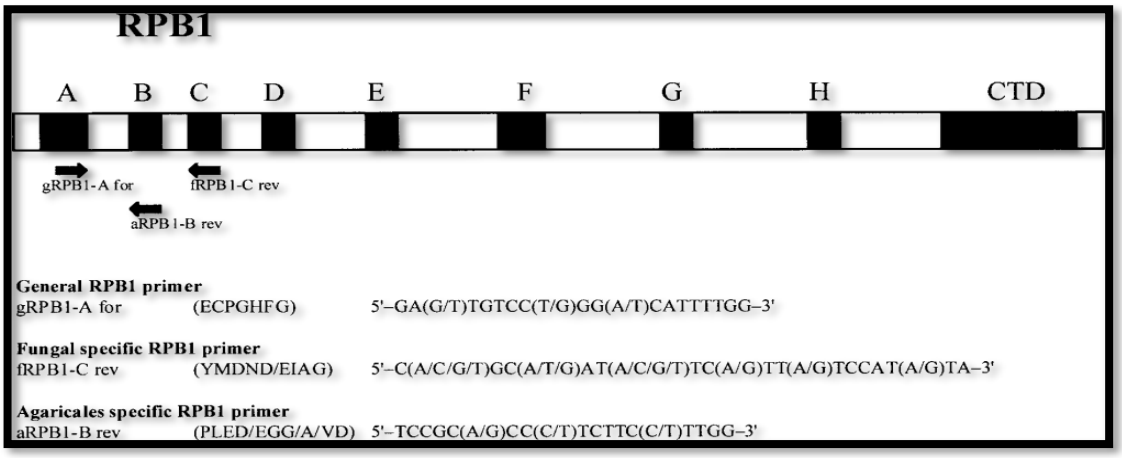


Diagram 10.24 RPB1 amplification and sequencing primers. Shaded boxes represent conserved amino acid motifs among Eukaryotes (Stefani, Jones & May 2014)

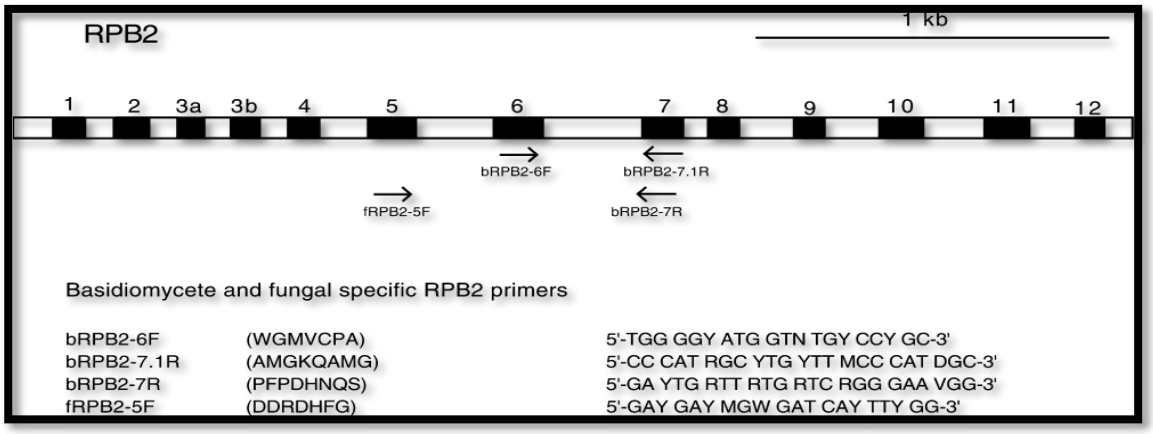


Diagram 10.25 RPB2 primer sequences.
Primer arrows are not drawn to scale
(Matheny et al. 2002) (Matheny & Ammirati 2003) (Matheny 2005)

*Our efforts have enabled the targeting of 6 primers that are appropriate candidates for fungal specific markers (Table 10.25.).

Table 10.25 Designed and used new and unique primers for the amplification of three regions (SSU/LSU/EF) of DNA in *Psilocybe* in my research:

Name	Sequence	Scale	Purification
SSU-SR6R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGWAAAAGTCGTAACAAGG	100nm	STD
ITS4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC	100nm	STD
LSU200-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACKGCGAGTGAAGMGG	100nm	STD
LSU-4,765 R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTGTTTCAAGACGGGTCGAT	100nm	STD
EF595F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGTGACTTCATCAAGAACATG	100nm	STD
EF1160R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGATCTTGTAGACGTCCTG	100nm	STD

- Primer (molecular size marker) combinations used is this study are;

Name: SSU/SR6R
Type: Primer Bind (primer bind) (Created by primer3)
Length: 20bp
Interval: 344 -> 363
Mismatches: 0
%GC: 35.0
Tm: 51.1 - 51.6
Hairpin Tm: None
Self-Dimer Tm: None
Pair Dimer Tm: None
Sequence: AAGWAAAAGTCGTAACAAGG
Degeneracy: 2
Product Size: 653bp

Name: ITS/ITS4
Type: Primer Bind (reverse) (primer-bind-reverse) (Created by primer3)
Length: 33bp
Interval: 1,101 -> 1,069 (996 -> 977)
Mismatches: 0
%GC: 45.0
Tm: 55.1
Hairpin Tm: None
Self-Dimer Tm: 0.2
Pair Dimer Tm: None
Sequence: TCCTCCGCTTATTGATATGC
Product Size: 653bp

Name: LSU200-F
Type: Primer Bind (primer-bind) (Created by primer3)
Length: 17bp
Interval: 4,029 -> 4,045 (52 -> 68)
%GC: 58.8
Tm: 57.6
Hairpin Tm: 45.9
Self-Dimer Tm: None
Pair Dimer Tm: None
Product Size: 597bp
Sequence: AACKGCGAGTGAAGMGG

Name: 4,765 R
Type: Primer Bind (reverse) (primer-bind-reverse) (Created by primer3)
Length: 20bp
Interval: 4,765 -> 4,746 (507 -> 526)
%GC: 50.0
Tm: 58.2
Hairpin Tm: 34.1
Self-Dimer Tm: None
Pair Dimer Tm: None
Sequence: GTGTTTCAAGACGGGTCGAT
Product Size: 597bp

Name: RPB1/Ps-int2F
Type: Primer Bind (primer bind) (Created by primer3)
Length: 19bp
Interval: 1,575 -> 1,593 (1,561 -> 1,578)
Mismatches: 0
%GC: 66.7
Tm: 60.8 - 61.8
Hairpin Tm: 51.2
Self-Dimer Tm: 10.0
Sequence: GGCWGAACGAGSAGTGCG
Degeneracy: 4

Name: 2,357 R
Type: Primer Bind (reverse) (primer_bind_reverse) (Created by primer3)
Length: 22bp
Interval: 2,357 -> 2,336 (980 -> 959)
%GC: 54.5
Tm: 61.0
Hairpin Tm: None
Self-Dimer Tm: None
Pair Dimer Tm: None
Sequence: CACTTCTGACGGGGTAATGAGC

Product Size: 600bp

Name: EF595F
Type: Primer Bind (primer bind) (Created by primer3)
Length: 21bp
Interval: 1 -> 21
%GC: 42.9
Tm: 55.3
Hairpin Tm: 39.7
Self-Dimer Tm: None
Sequence: CGTGACTTCATCAAGAACATG

Name: EF1160R
Type: Primer Bind (primer bind) (Created by primer3)
Length: 20bp
Interval: 1 -> 20
%GC: 55.0
Tm: 57.5
Hairpin Tm: None
Self-Dimer Tm: 13.0
Sequence: CCGATCTTGTAGACGTCCTG

Tables 10.26 (a-b-c-d-e-f-g-h) Oligo Tables

Tables 10.27 (a-b-c) New and unique designed RPB primers that they did not work for this research

They could not amplify any region or even part of the DNA.

Table 10.27a. Designed novel RPB primers that did not work for this research

Name	Sequence	Scale	Purification
RPB1/Ps-int2F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGCWGAACGAGSAGTGCG	100nm	STD
RPB1/2,357 R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCACTTCTGACGGGGTAATGAGC	100nm	STD

Table 10.27b. Designed novel RPB primers that did not work for this research

Name	Sequence	Scale	Purification
RPB1/1,554 F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGGATGCGTGGTGAAATGG	100nm	STD

Table 10.27c. Designed novel RPB primers that did not work for this research

Name	Sequence	Scale	Purification
RPB1/Ps-Ex2R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCGTAYTCTTCCGAGAGACC	100nm	STD

10.7 DNA amplification by Polymerase Chain Reaction (PCR)

The Polymerase chain reaction (PCR) is useful for detecting DNA Polymorphism (Lee, Cole & Linacre 2000a). PCR is the base of most molecular approaches involved in diversity estimations. However, DNA from environmental samples contains PCR inhibitors and contaminants that interfere with PCR reactions (e.g., humic acid from soil). In countless cases, there can be differential amplification, loss of DNA following purification, production of PCR artefacts, and contamination. The quality of sequence data recovered can be extremely variable due to contaminating background sequences. Because of the risk of cross-contamination from handling of many identical PCR reactions, it is obligatory to follow severe precautions. Many of these precautions purely require good sterile technique and wakefulness of potential contamination sources. Concentration of purified amplicons is determined by Nano-drop spectrophotometer. Negative controls (omitting DNA template) are also included in each PCR experiment (Vilgalys & Hester 1990).

PCR techniques targeting the rDNA region are simple, rapid and quite useful tools for the identification of most fungi. DNA extracted from environmental samples is amplified with a primer pair (precise to the groups of organisms under investigation and one of them attached to a GC clamp) and then purified PCR samples are separated electrophoretically through a gradient of increasing chemical grade. Based on the melting behaviour, different sequences migrate at different loci, generating different banding patterns where each presumably represents a microbial taxon. The bands can then be removed from the gel and processed (either by creation of clone libraries and screening clones, or reamplified and sequenced) to find phylogenetic sequence information on singular microbial members of the microbial community. (TA: Annealing temperature: the temperature that primers bind to DNA, TM: Melting Temperature: 50% of the helicase are dissociated). PCR amplification of chimeric sequences is not unusual. Sequence analyses of these typically indicate that they are not phylogenetically related to other known fungi, as they occupy unique spot in the phylogenetic tree. In these cases, one will wrongly assume that these

sequences represent novel taxa that escape microscopic or cultural detection (Jeewon & Hyde 2007).

Therefore, morphology characteristics study is suitable for advance analysis method on genetic study of fragment size and efficient removal of primer-dimers or other small DNA fragments are verified using a Bioanalyzer (Agilent Technologies, Inc., CA, and USA). In addition to a standard PCR, using universal primers, we also use the Agilent Bioanalyzer (Palo Alto, CA, USA) to visualize the quantity of DNA in each fragment size. The sizes of DNA are determined by micro capillary electrophoresis using the Agilent Bioanalyzer. This technology uses a microfluidics-based platform for sizing, quantification and quality control of DNA (http: //www. home.agilent.com) and it has a potential of further application, such as evaluating quality and quantity of total RNA. By using this system, we can assess degrees of DNA fragmentation more factually than using a standard gel electrophoresis (Hosaka, K & Uno 2013). To facilitate retrieval of sequences obtained from individual specimens, we tag individual specimens during PCR amplification using unique 10-mer oligonucleotides attached to DNA barcoding PCR primers. Next-generation sequencing is of great value because of its protocol simplicity, greatly reduced cost per barcode read, faster throughout and added information content. The bioassay of the methodology is the use of molecular techniques centred on 3 amplified regions in this study. Efforts are made to PCR amplify parts (600 bp)

of three genetic loci that is useful in resolution of *Psilocybe* taxa. The PCR base trial is simple to practice and is conclusive for the genera. It is perfect if there is a limited or degraded sample (Linacre, A, Cole & Lee 2002). To enable recovery of sequences obtained from single specimens we label individual specimens all through PCR amplification by unique 10-mer oligonucleotides to DNA barcoding PCR primers (Shokralla et al. 2014).

DNA templates from all 96 specimens are PCR amplified with the tagged and fusion-tailed primers in the identical reaction conditions. Miseq is used for specimen-based barcodes since it offers high-quality 300-bp Paired-End (PE) reads. Direct PCR escapes DNA extraction, because it uses a small piece of tissue as templates with the success rate of 89%. Direct PCR established based

on a polymerase chain reaction (PCR) ought to be used only for taxa with large numbers of right similar specimens but PCR amplification is a basic type of PCR clean-up followed by Illumina Sequencing. In a following trial we use the resu lts of the first experiment to encourage more simplifications. For verification and quantification of PCR products we use gel electrophoresis. Also use uniquely tagged forward and reverse primers for PCR reactions (Meier et al. 2016).

PCR products from the same samples is pooled and cleaned using Gel and PCR clean up system (Větrovský et al. 2016). PCR products will be normalized with Qubit fluorimeter with the dsDNA HS (High Sensitivity) Kit and AMPured magnetic beads is used for purifying libraries and challenges (Nilsson et al. 2019). The ITS, SSU, LSU and EF are amplified by PCR using the designed primer sets to afford the amplicon in lengths of 600 bp, respectively.

10.8 Normalization

Table 10.28 Qbit Results for all 96 samples

3.730	0.918	2.350	2.350	9.600	242.000	141.000	366.000	37.300	38.100	0.354	36.600
5.850	7.970	0.989	0.509	3.130	40.700	193.000	108.000	73.500	51.000	191.000	55.000
13.300	11.300	49.600	1.770	48.800	49.900	47.900	54.000	319.000	55.000	0.797	55.000
36.900	59.000	6.030	2.940	44.100	205.000	260.000	206.000	57.000	46.600	44.400	43.300
6.360	36.400	56.000	5.490	414.000	42.800	78.300	55.000	45.800	42.500	48.200	41.400
48.100	55.000	1.700	59.000	352.000	53.000	53.000	60.000	202.000	44.400	3.720	53.000
35.700	3.770	8.990	6.030	57.000	379.000	40.300	44.000	1.500	0.415	36.100	49.400
0.355	47.500	40.300	19.900	31.100	374.000	59.000	47.600	28.500	18.700	57.000	53.000
9.419	2.318	5.934	5.934	24.242	611.098	356.053	924.223	94.190	96.210	0.894	92.422
14.772	20.126	2.497	1.285	7.904	102.776	487.364	272.722	185.602	128.785	482.313	138.886
33.585	28.535	125.250	4.470	123.230	126.007	120.957	136.361	805.539	138.886	2.013	138.886
93.180	148.987	15.227	7.424	111.361	517.666	656.552	520.191	143.936	117.674	112.119	109.341
16.060	91.917	141.411	13.863	1045.433	108.079	197.723	138.886	115.654	107.321	121.715	104.543
121.462	138.886	4.293	148.987	888.870	133.836	133.836	151.512	510.090	112.119	9.394	133.836
90.150	9.520	22.702	15.227	143.936	957.051	101.766	111.109	3.788	1.048	91.160	124.745
0.896	119.947	101.766	50.251	78.534	944.425	148.987	120.200	71.968	47.221	143.936	133.836
8.493	34.511	13.481	13.481	3.300	0.131	0.225	0.087	0.849	0.832	89.493	0.866
5.415	3.975	32.033	62.241	10.122	0.778	0.164	0.293	0.431	0.621	0.166	0.576
2.382	2.804	0.639	17.899	0.649	0.635	0.661	0.587	0.099	0.576	39.750	0.576
0.859	0.537	5.254	10.776	0.718	0.155	0.122	0.154	0.556	0.680	0.714	0.732
4.981	0.870	0.566	5.771	0.077	0.740	0.405	0.576	0.692	0.745	0.657	0.765
0.659	0.576	18.636	0.537	0.090	0.598	0.598	0.528	0.157	0.714	8.516	0.598
0.887	8.403	3.524	5.254	0.556	0.084	0.786	0.720	21.120	76.339	0.878	0.641
89.241	0.667	0.786	1.592	1.019	0.085	0.537	0.666	1.112	1.694	0.556	0.598

Qubit Results

(concentration in ng/µl)/
(660 g/mol × average library size)
× 10^6 = concentration in nM

C1×V1= C2×V2
concentration in nM × Volume of Tris Buffer = 4×20

Figures 10.3 (a-b) Some examples of normalized results running on the gel

Figure 10.3a. Some examples of normalized results on the gel (the first lane from left shows the DNA ladder bands, lanes 2 and 3 are samples)

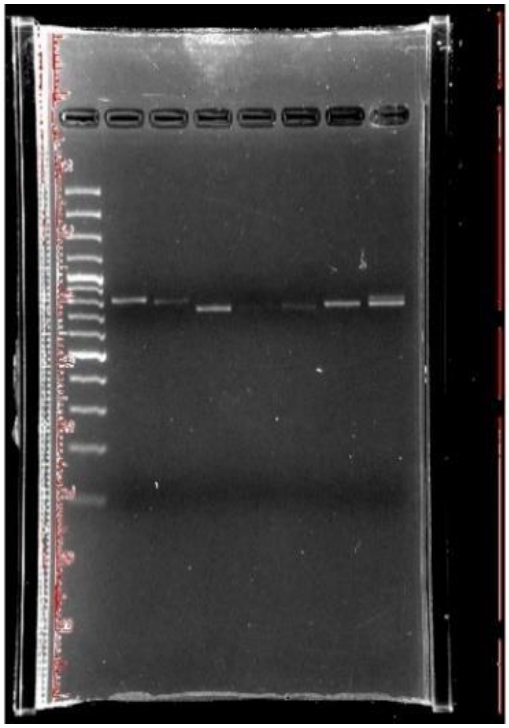


Figure 10.3b. Some examples of normalized results on the gel (the first lane from left shows the DNA ladder bands, lanes 2-8 are samples)

DNA samples extracted from the original specimens is used as a template for PCR amplification. All PCR reagents and Taq polymerase is obtained and used as recommended by the supplier (Takara Shuzo, Japan). Briefly, a total of 0.2-lmol of the appropriate primer set and adequate amounts of template DNA is used in each 20 ul reaction mixture. In contrast, adequate amount of sterile distilled water is added for serving as a negative control. PCR amplification is performed with a T100 Thermal Cycler (BIO-RAD). Thereafter, amplified DNA products is electrophoresed on 1% agarose gels dissolved in Tris-Borate-EDTA (TBE) buffer and visualized under ultraviolet (UV) light after staining with ethidium bromide (Figure 10.3.). A DNA ladder (1-kb plus, catalogue no. 10787-018, Invitrogen, Taipei, Taiwan) (Figure 10.4.) is used as the standard marker for comparison (Always thaw RNase / DNA ladder / DNA loading dye in the room temperature).

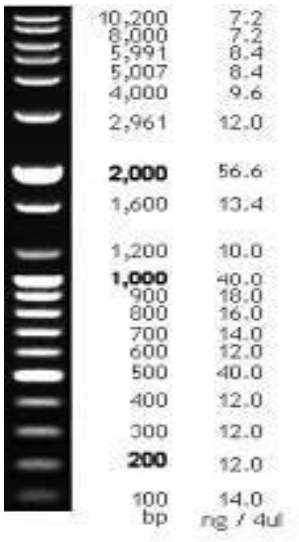


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Figure 10.4 DNA Ladder (bp units)

SYBR Green is used to screen the accumulation of the amplified product during PCR and subsequent product melting in the Illumina. PCR is performed in a total volume of 25 μL containing 1.0 μL DNA template, 1.0 μL of each forward and reverse primers (10 μmol/L), 12.5 μL 2 × Master Mix (Tiangen Biotech Co.

Ltd., Beijing, China) and 10.5 μL ddH2O, using the following parameters: The reactions is carried out in a Thermal Cycler under the specific temperature regime. The annealing temperature dependent on the gene amplified, the melting temperature is calculated using the formula based on the nearest neighbour thermodynamic theory. It is the temperature at which half of the oligonucleotides are bonded.

GC content × 4 = x (GC% is the percentage of G and C in the primer. It is calculated by dividing the sum of G and C with the total number of bases present in the primer) AT content × 2 = y
X + Y = TM (Table 10.29.)

Table 10.29 Suggested and accepted TM for current study designed primers

Primers	Suggested TM °C
SSU-SR6-R	49.14 or 51.1 or 51.6
ITS4	55.1 or 55.59
LSU 200-F	57.6 or 59.13
LSU-4,765-R	57.12 or 58.2
EF595-F	53.57 or 55.3
EF 1160-R	55.33 or 57.5

Prior to sequencing, amplification of the three region is performed using six Primers. The aliquots of PCR products are purified using a Gel Extraction and PCR Purification (Agencourt AMPure XP) by following the manufacturer's instructions to remove all excess primers. The purified products are sequenced in both forward and reverse directions using Illumina MiSeq sequencing (Lower throughput, bench-top version sequencing machine). Amplification is yielded a strong band of about 600 bp. Sequences is compared with accessions in the GenBank database via BLAST (Basic Local Alignment Search Tool) searching to obtain the most likely taxonomic designation. Molecular data is generated for three loci: the internal transcribed spacer (ITS), nuclear large subunit (nucLSU), and (EF). Primers used for amplifying the three loci are listed in Table 30. The concentrations of everything check with Nano-drops.

1% Agarose Gel Method for DNA Extracts (2% Agarose Gel for PCR Products)

0.5 gm Agarose Powder

50 ml 1×TAE Buffer

3µL Syber Safe

For every 5 µL of samples, 1 µL of Loading Dye

5 µL Thermo-Scientific DNA (DNA Ladder)

1) Prepare Gel

Combine Agarose and TAE in a conical flask, cover with Glad wrap, Microwave gently until all agarose is dissolved (1 Min + 10 Sec), Allow to cool slightly, add Ethidium Bromide

2) Prepare the tray

Using Masking tape close the ends of the tray then lay it on a sheet of paper towel, Place comb in the tray around the 1cm mark, pour mixture into tray to set roughly 15 Min.

3) Prepare the samples

Collect 7 clean micro tubes and label their lids carefully, Pipette 10 µL of sample and loading dye into the tubes, give the tubes a quick centrifuge.

4) Loading the gel, once the gel is set and samples are prepared.

Fill the gel bath with 1×TAE buffer, Remove the comb from the gel/remove the tape, place gel into bath.

Carefully pipette Thermo-scientific DNA into the first lane (5 µL is enough) or 6µLof (100bp + loading Dye + Water)

Carefully pipette samples into the remaining lanes taking care to record which sample is in which lane

Once gel is loaded, turn the power on to 80 (for each 1 cm 4-10 voltage)

Check the power is on by looking for little bubbles on either end of the bath.

Leave to run for 30 min (until 2/3 of the length of the gel)

5) once gel is run

Carefully remove gel from the bath, the gel can stay in the tray Assess gel with the UV camera.

- PCR stages based on the pictures; (Figures 10.5)

Figures 10.5 (a-b-c-d-e-f-g-h-i-j-k-l) Protocol stage 1, 2, 3, 4, 5 of PCR

Figure 10.5a. Stage one of PCR protocol

Figure 10.5b. Stage one of PCR protocol

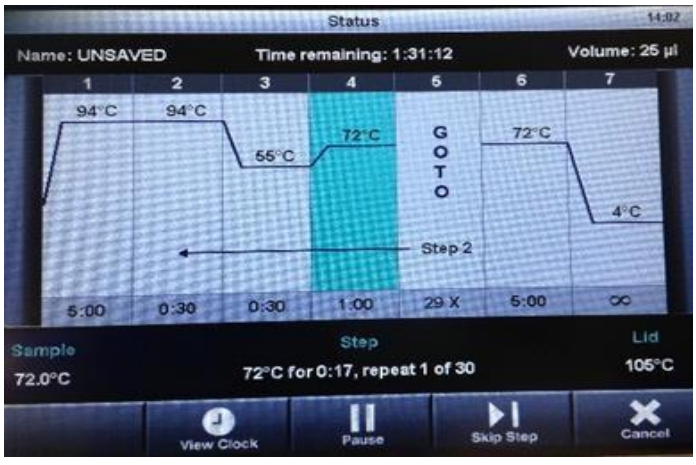


Figure 10.5c. Stage one of PCR protocol

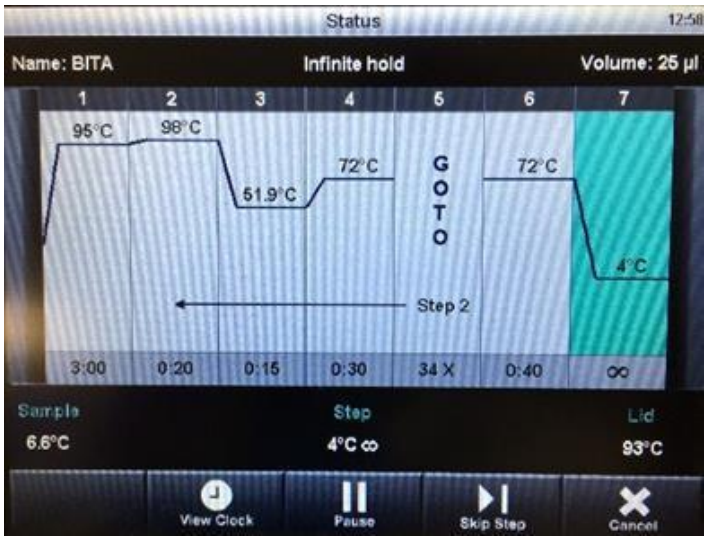


Figure 10.5d. Stage one of PCR protocol

Figure 10.5e. Stage two of PCR protocol

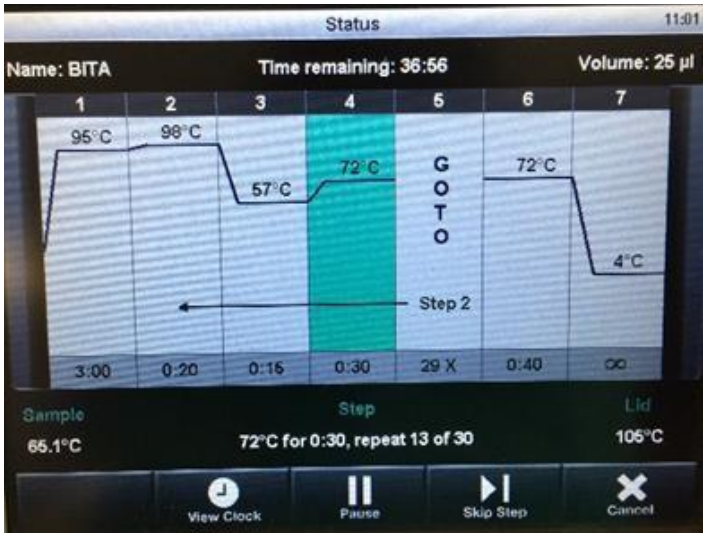


Figure 10.5f. Stage two of PCR protocol

Figure 10.5g. Stage two of PCR protocol

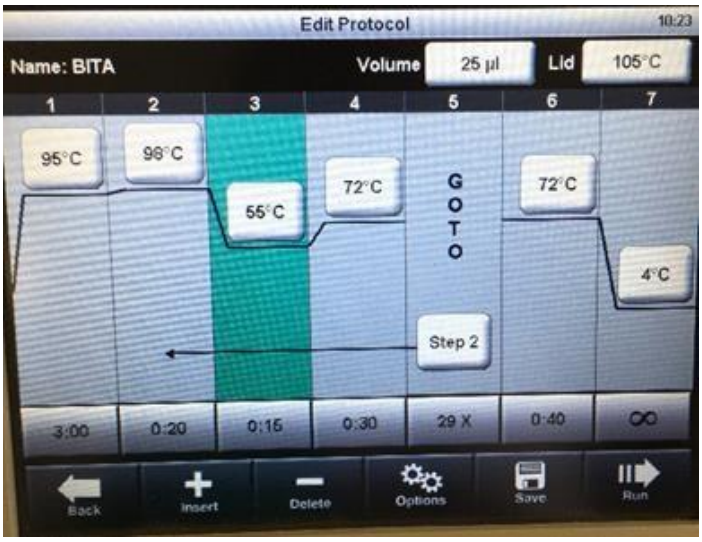


Figure 10.5h. Stage two of PCR protocol

Figure 10.5i. Stage three of PCR protocol

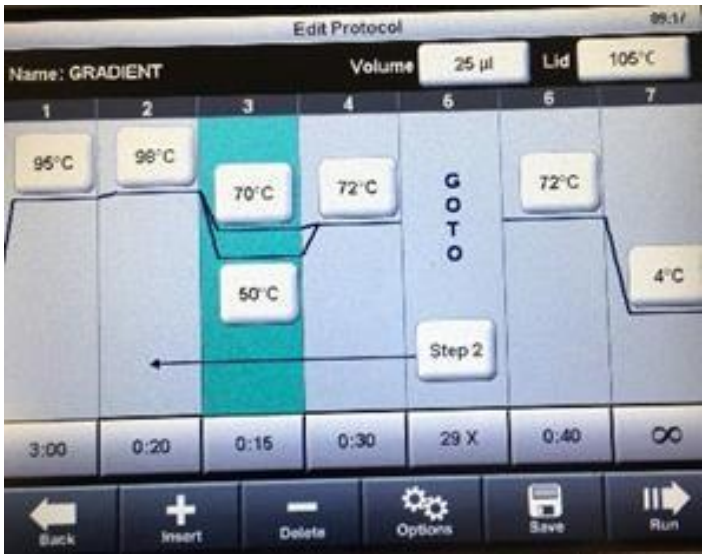


Figure 10.5j. Stage three of PCR protocol

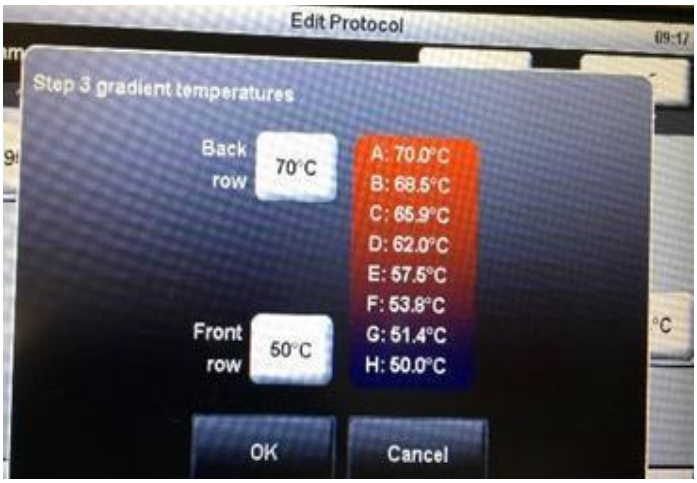


Figure 10.5k. Stage four of PCR protocol

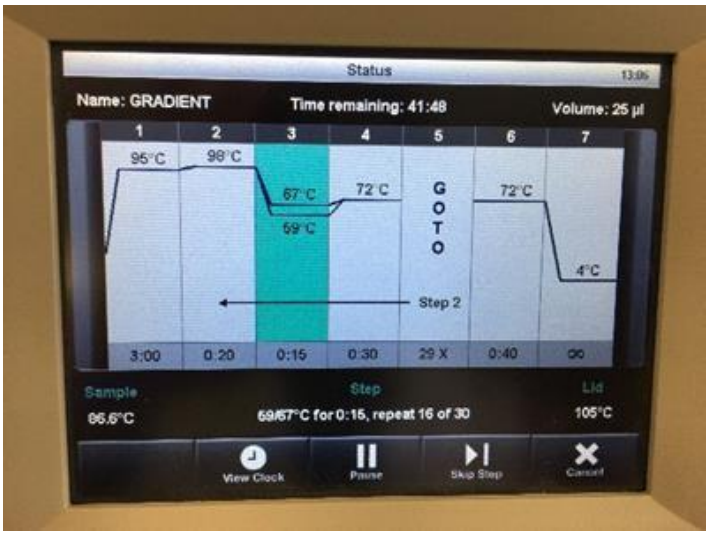


Figure 10.5l. Stage four of PCR protocol

10.9 Bioanalyser stage (Pre-Index DNA Assay)

Table 10.30 Bioanalyser results for 10 random samples

Library		2305193		2362271		2317392		1061294		2305213		2341799		2305258		2264832		2317408		2192210	
Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]
35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	27	0.00
715	513.34	789	807.77	731	230.94	507	6.16	741	714.40	733	522.86	750	280.87	756	287.77	738	528.46	726	418.26	32	0.00
766	770.61	971	905.14	788	432.43	515	5.12	794	565.45	783	598.74	794	455.23	801	383.53	792	487.61	783	436.98	35	125.00
910	679.45	1,999	34.58	940	390.08	540	11.13	904	1264.11	896	1544.12	930	657.16	922	898.12	911	1025.83	906	899.47	48	39.89
1,808	7.74	4,134	30.84	1,532	19.57	733	614.16	2,134	9.85	2,122	22.26	1,937	10.23	1,880	30.04	1,940	16.58	1,825	16.95	61	45.47
1,908	7.75	4,795	16.12	1,732	15.25	788	431.41	2,369	9.09	5,494	72.33	2,006	14.92	2,318	13.28	3,675	48.57	3,427	12.80	67	24.80
1,973	7.18	5,529	16.80	2,640	4.64	923	1126.62	2,729	12.04	5,865	73.10	2,404	14.98	4,921	46.68	4,639	18.92	5,000	25.76	84	39.95
2,173	8.23	5,933	53.74	2,904	5.01	1,981	9.60	3,229	14.19	10,380	75.00	3,763	31.75	5,526	15.10	5,910	58.62	5,875	11.42	91	31.84
3,104	8.87	10,380	75.00	3,507	9.25	2,355	8.03	4,595	12.76	13,389	0.00	4,741	16.06	6,088	34.18	10,380	75.00	7,376	11.07	100	37.78
3,688	8.15	13,308	0.00	4,788	5.78	2,506	6.96	5,238	11.14	15,604	0.00	5,676	20.95	10,380	75.00	16,403	0.00	10,380	75.00	119	40.95
5,332	12.76	14,822	0.00	5,315	9.42	2,778	6.28	6,444	18.80	16,682	0.00	7,867	8.28	14,598	0.00			13,685	0.00	147	31.97
5,770	9.70	15,580	0.00	6,671	6.01	3,905	14.89	7,893	13.40	17,648	0.00	10,380	75.00	15,430	0.00			15,067	0.00	156	42.88
7,116	8.89	17,145	0.00	7,998	4.30	4,954	13.30	10,380	75.00	21,565	0.00	14,295	0.00	16,856	0.00			16,269	0.00	190	45.34
7,970	9.77	19,568	0.00	10,380	75.00	5,808	10.18	13,530	0.00	37,520	0.00	15,931	0.00	19,827	0.00					227	114.50
10,380	75.00			13,073	0.00	6,507	16.08			38,996	0.00									238	43.65
13,493	0.00					7,817	4.55			40,813	0.00									251	44.49
15,702	0.00					8,405	3.96			41,608	0.00									261	29.98
17,911	0.00					10,380	75.00													287	28.56
																				300	31.03
																				314	37.74
																				328	37.58
																				346	36.38
																				380	31.51
																				425	51.54
																				472	90.32
																				486	32.65
																				520	31.97
																				773	587.99
																				901	459.14
																				1,911	12.13
																				2,371	3.05
																				5,114	43.80
																				5,597	14.51
																				6,518	18.26
																				10,380	75.00
																				15,750	0.00
																				18,525	0.00
																				20,274	0.00
																				21,722	0.00
																				22,929	0.00
																				26,308	0.00
																				28,299	0.00
																				30,833	0.00
																				33,487	0.00
																				34,996	0.00
																				36,685	0.00
																				37,831	0.00
																				39,400	0.00
																				41,873	0.00
																				42,778	0.00

2305193		2362271		2317392		1061294		2305213		2341799		2305258		2264832		2317408		2192210	
Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]	Size [bp]	Conc. [pg/µl]
35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00	35	125.00
95	31.04	650	9137.49	470	247.36	470	10.23	660	6759.25	414	8.48	475	13.56	292	19.74	435	7.96	496	5.64
355	5.60	840	1102.11	612	188.23	632	648.99	825	4277.74	446	29.38	637	526.58	342	48.32	470	14.47	546	6.36
446	5.11	2,141	44.08	633	2437.52	660	3913.76	959	762.05	576	11831.62	658	6586.59	362	157.95	508	6.98	633	581.67
458	8.74	2,931	106.83	658	4810.70	825	1070.97	1,855	41.24	592	15204.06	796	4912.22	372	33041.75	526	6.11	657	4404.64
630	174.29	3,523	33.58	826	1554.88	960	961.17	2,382	52.69	667	12186.65	4,872	146.25	375	39445.20	552	7.25	788	1808.59
647	11265.45	4,209	37.77	915	280.51	1,571	25.96	2,621	24.44	6,896	20.49	5,424	146.40	397	28812.33	578	11.29	1,255	25.91
830	9061.81	4,895	237.34	1,057	726.14	1,961	23.33	2,758	100.65	10,380	75.00	10,380	75.00	755	22.83	612	104.67	1,515	40.17
5,023	361.91	10,380	75.00	1,931	52.84	2,760	38.51	4,773	44.85	12,245	0.00	13,032	0.00	1,147	15.70	634	1656.80	1,590	45.46
10,380	75.00	12,658	0.00	2,078	26.12	3,618	17.94	6,472	13.56	14,537	0.00	14,103	0.00	1,245	16.82	658	6894.29	1,841	27.18
11,819	0.00	14,379	0.00	2,636	104.47	4,171	16.00	10,380	75.00	15,550	0.00	15,796	0.00	1,383	24.76	774	6461.75	2,217	25.99
13,953	0.00	16,251	0.00	4,698	19.88	4,882	38.84	16,072	0.00	16,456	0.00	17,206	0.00	1,468	24.99	2,994	59.52	2,840	34.36
14,698	0.00	18,023	0.00	5,668	12.77	6,147	53.55	31,752	0.00	18,375	0.00	19,350	0.00	1,560	24.09	3,332	25.96	3,790	23.85
15,690	0.00	19,238	0.00	6,598	10.06	10,380	75.00	33,954	0.00	19,441	0.00	29,674	0.00	1,685	20.39	3,897	142.10	4,699	18.94
16,633	0.00	20,351	0.00	8,011	2.59	13,214	0.00	37,444	0.00	19,920	0.00	30,972	0.00	1,855	9.65	8,476	10.61	5,564	29.54
18,221	0.00			10,380	75.00					21,679	0.00	32,890	0.00	1,973	9.99	10,380	75.00	7,852	15.49
19,760	0.00									22,479	0.00	33,849	0.00	2,735	5.36	12,111	0.00	10,380	75.00
21,149	0.00									23,971	0.00	36,049	0.00	4,449	8.96	13,265	0.00	13,023	0.00
22,341	0.00									25,517	0.00	38,193	0.00	5,273	11.39	15,284	0.00	16,931	0.00
										26,849	0.00			5,608	13.47	16,265	0.00	18,310	0.00
										27,435	0.00			6,219	14.52	20,072	0.00		
										29,781	0.00			6,646	16.92	20,592	0.00		
														7,503	27.42	32,707	0.00		
														8,597	38.23	33,861	0.00		
														9,286	29.53	37,092	0.00		
														10,380	75.00	38,130	0.00		
														12,123	0.00	39,457	0.00		
														13,663	0.00				
														15,040	0.00				

Figures 10.6 (a-b-c-d-e-f-g) Bioanalyser results running on the gel

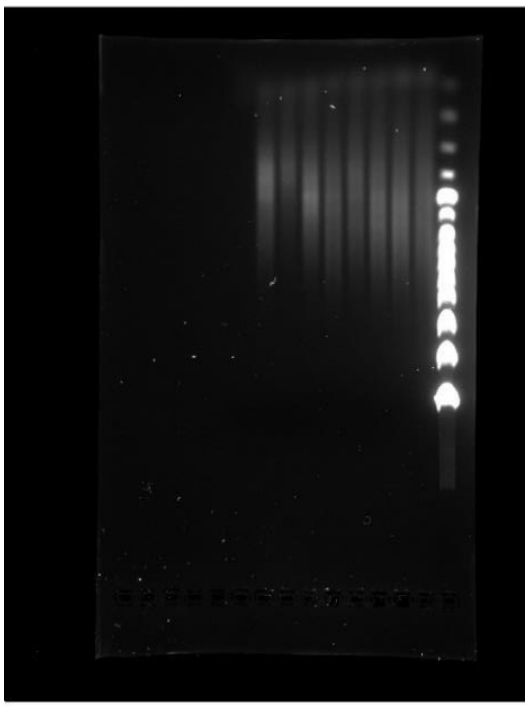


Figure 10.6a. Bioanalyser results on the gel (the first lane from right shows the DNA ladder bands, the next 8 lanes are samples)

Figure 10.6b. Bioanalyser results on the gel (the first lane from left shows the DNA ladder bands, the next 4 lanes are samples)

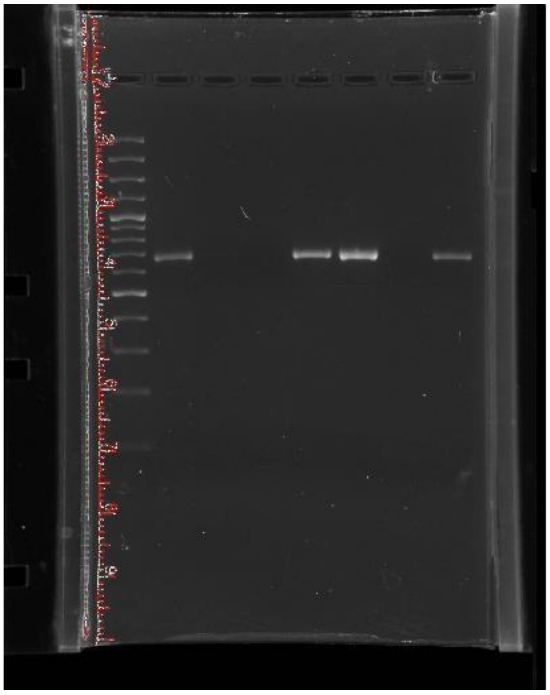


Figure 10.6c. Bioanalyser results on the gel (the first lane from left shows the DNA ladder bands, the next 7 lanes are samples)

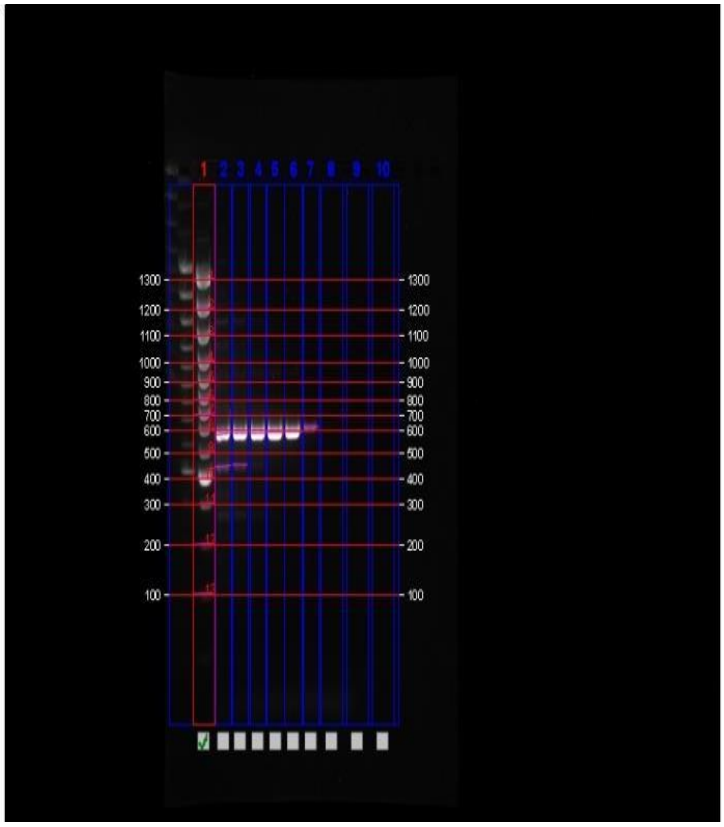


Figure 10.6d. Bioanalyser results on the gel (the first lane from left shows the DNA ladder bands, the next 7 lanes are samples)

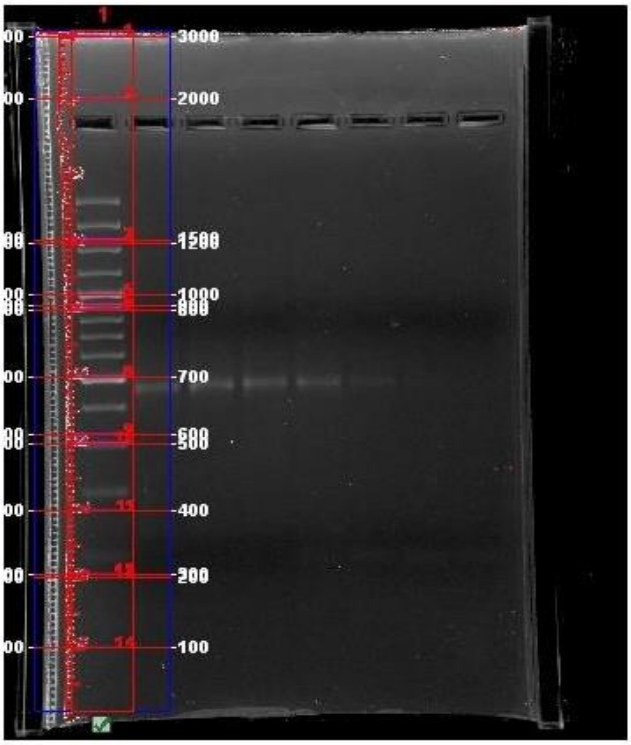


Figure 10.6e. Bioanalyser results on the gel (the first lane from left shows the DNA ladder bands, the next 7 lanes are samples)

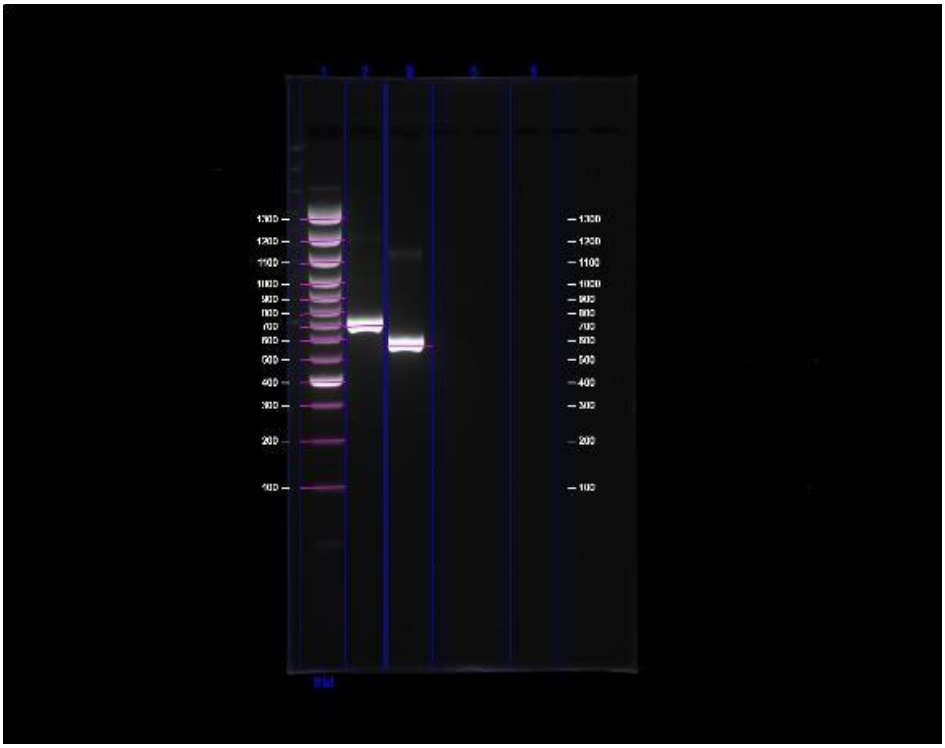


Figure 10.6f. Bioanalyser results on the gel (the first lane from left shows the DNA ladder bands, the next 2 lanes are samples)

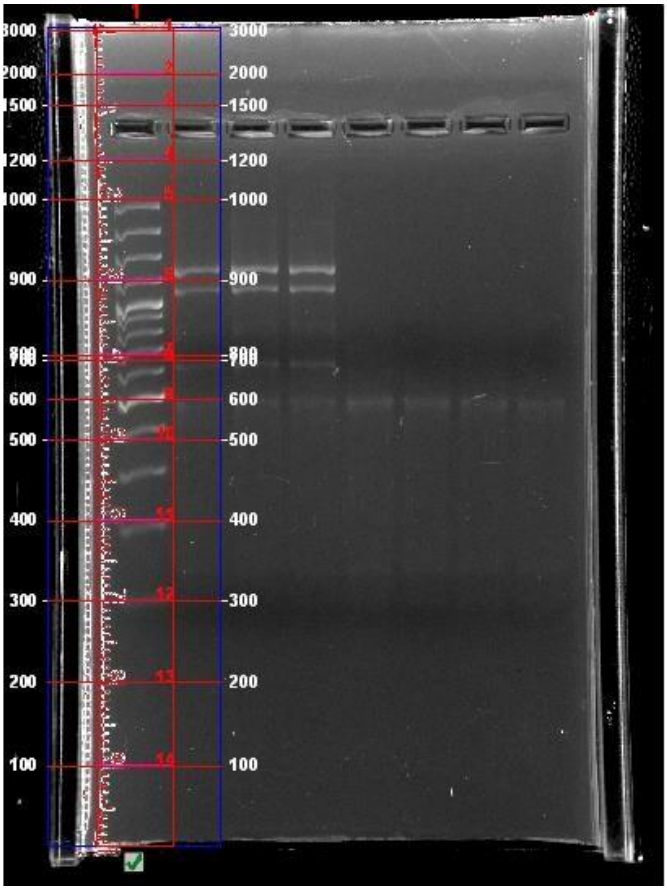


Figure 10.6g. Bioanalyser results on the gel (the first lane from left shows the DNA ladder bands, the next 7 lanes are samples)

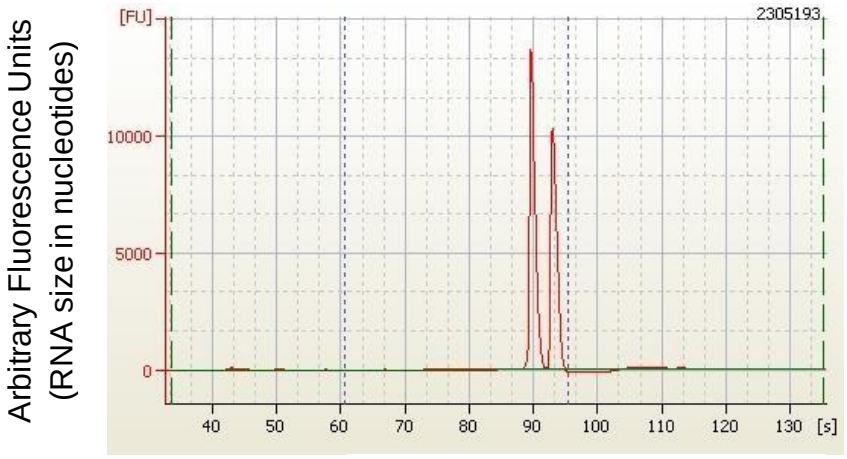


Diagram 10.26 Bioanalyser graph for one example (sample No.2305193)

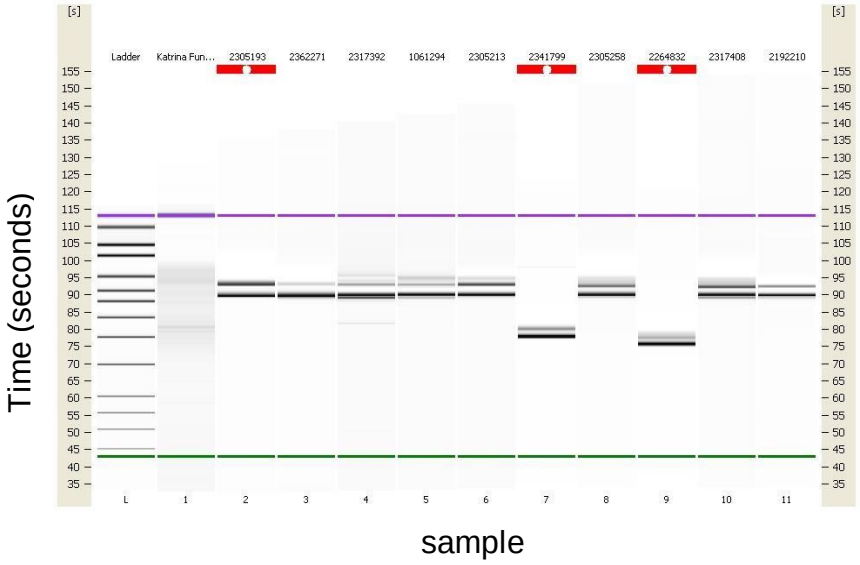


Diagram 10.27 Bioanalyser results for 10 random samples

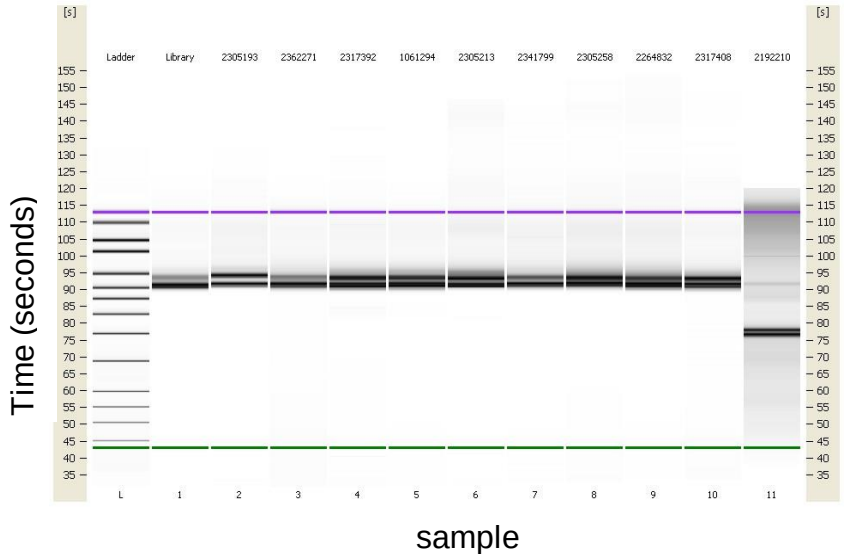


Diagram 10.28 Compared Bioanalyser results to library for 10 random samples

10.10 Indexing

Figures 10.7 (a-b) Indexing process/ through PCR

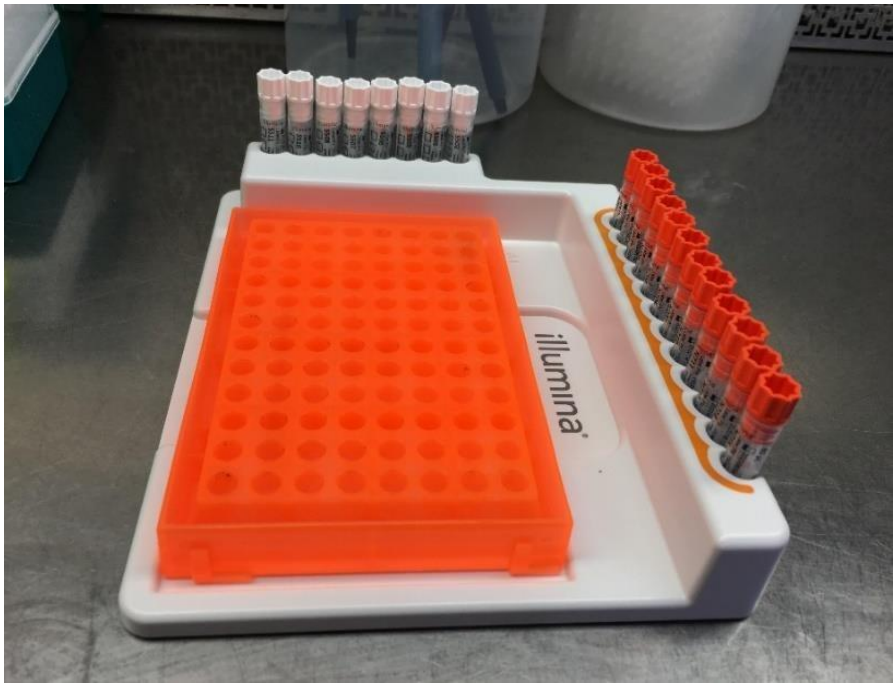


Figure 10.7a. Indexing process (first step)

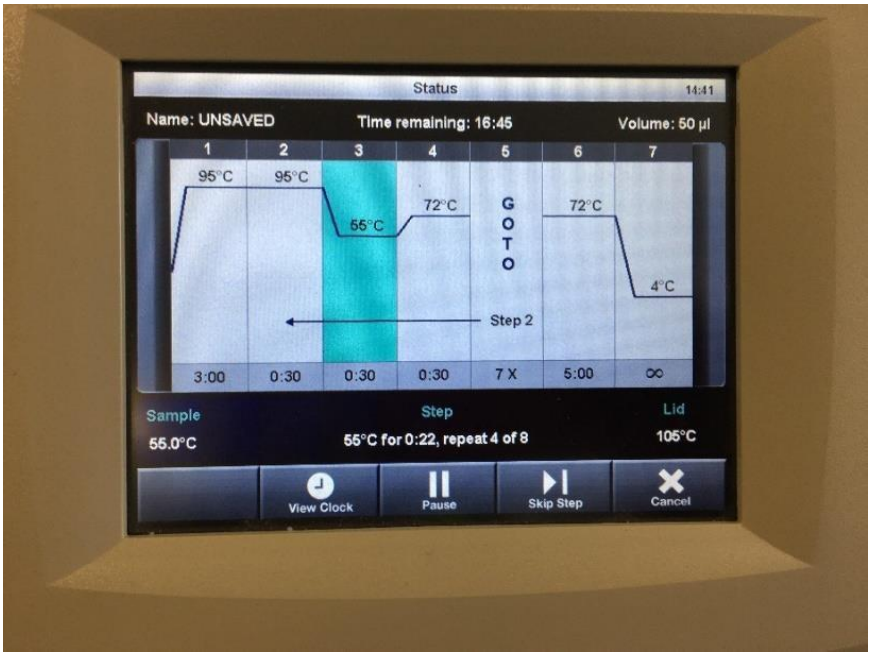


Figure 10.7b. Indexing procedure through PCR

Table 10.31 Quantification of prepared library by QPCR

Library #	Sample name	Dilution	Cq	Average fragment length (bp)	Outliers / outside curve	Average Cq	Difference	Delta Cq	log (concentration)	Average concentration (pM)	Size-adjusted concentration (pM)	Concentration of undiluted library (pM)	Concentration of undiluted library (nM)	Concentration of undiluted library (ng/µL)	%Deviation	Working concentration (pM)	Working concentration (nM)	Working concentration (ng/µL)
1	Homa Library	10000	15.14	750		15.15	0.00	1.4	-0.13	0.74	0.44	4,442	4.44	2.06	21.2%	3,971	4.0	1.8
					16.06													
			15.15				0.01											
		20000			17.58	16.51			-0.54	0.29	0.17	3,500	3.50	1.62				
			16.82				0.31											
			16.20				-0.31											
2	pHX	10000	14.08	500		14.03	0.05	#DIV/0!	0.20	1.58	1.43	14,294	14.29	4.42	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
			13.95				-0.08											
			14.05				0.02											
						#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!				
							#DIV/0!											
							#DIV/0!											

Table 10.32 Details of KAPA HiFi Hot-Start Ready-Mix PCR Kit

Step	Temperature °C	Duration	Cycles
Initial denaturation1	95	180 sec	1
Denaturation2	98	20 sec	15 – 356
Annealing3,4	60 – 75	15 sec	
Extension5	72	15 – 60 sec/kb	
Final extension	72	60 sec/kb	1

Component	50 µl rxn1	Final conc.
PCR-grade water	Up to 50 µl	N/A
2X KAPA HiFi HotStart	25.0 µl	1X
ReadyMix2, 3		
10 µM Forward Primer	1.5 µl	0.3 µM
10 µM Reverse Primer	1.5 µl	0.3 µM
Template DNA4	As required	As required

Table 10.33 Kappa Titration details for QPCR

Well	Well Type	Template	Ct (dR)	Quantity (picomoles)	DF	Length (bp)	Kapa (bp)	Raw value	Adj. Conc. (nM)	Avg. Conc. (nM)	SD	CV (%)		Adj. Conc. (nM) = (DF*Quantity)/1000)*(Kapa/Length)
A4	Unknown	Pool1_R1	1.40E+01	1.21E-01	500000	200	452	60.25	136.17	65.98	61.11	92.63		CV(%)=(SD/average)*100
A5	Unknown	Pool1_R2	1.57E+01	3.29E-02	500000	200	452	16.465	37.21					
A6	Unknown	Pool1_R3	1.62E+01	2.17E-02	500000	200	452	10.865	24.55				72.83144348	
B4	Unknown	Pool1_R1	1.32E+01	2.32E-01	5000000	260	452	1158.5	2014.01	81.46	84.55	103.79		
B5	Unknown	Pool1_R2	16.6	1.63E-02	5000000	260	452	81.25	141.25					
B6	Unknown	Pool1_R3	19.03	2.49E-03	5000000	260	452	12.47	21.68					
A1	Standard	1	22.43	5.30E-04										
A2	Standard	1	24.97	9.13E-05										
A3	Standard	1	24.56	1.21E-04										
B1	Standard	2	6.92	2.00E+01										
B2	Standard	2	7.02	2.00E+01										
B3	Standard	2	7.03	2.00E+01										
C1	Standard	3	10.64	2.00E+00										
C2	Standard	3	10.78	2.00E+00										
C3	Standard	3	10.83	2.00E+00										
D1	Standard	4	13.82	2.00E-01										
D2	Standard	4	13.82	2.00E-01										
D3	Standard	4	13.88	2.00E-01										
E1	Standard	5	17.25	2.00E-02										
E2	Standard	5	17.37	2.00E-02										
E3	Standard	5	17.56	2.00E-02										
F1	Standard	6	20.43	2.00E-03										
F2	Standard	6	20.4	2.00E-03										
F3	Standard	6	20.63	2.00E-03										
G1	NTC	NTC	23.42	2.00E-04										
G2	NTC	NTC	23.77	2.00E-04										
G3	NTC	NTC	23.93	2.00E-04										

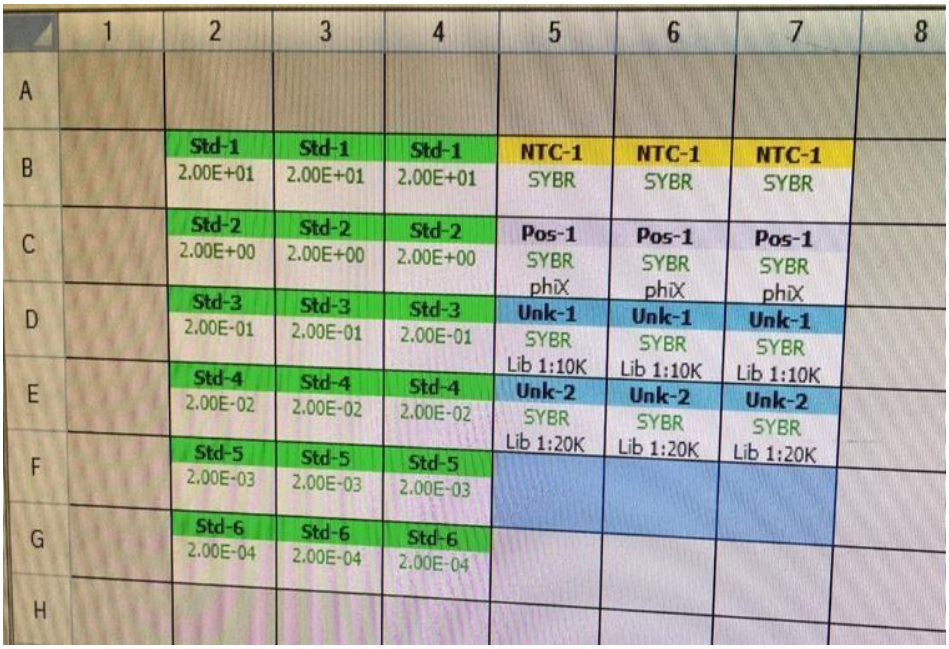


Figure 10.8 QPCR details (SYBR Safe & PhiX)

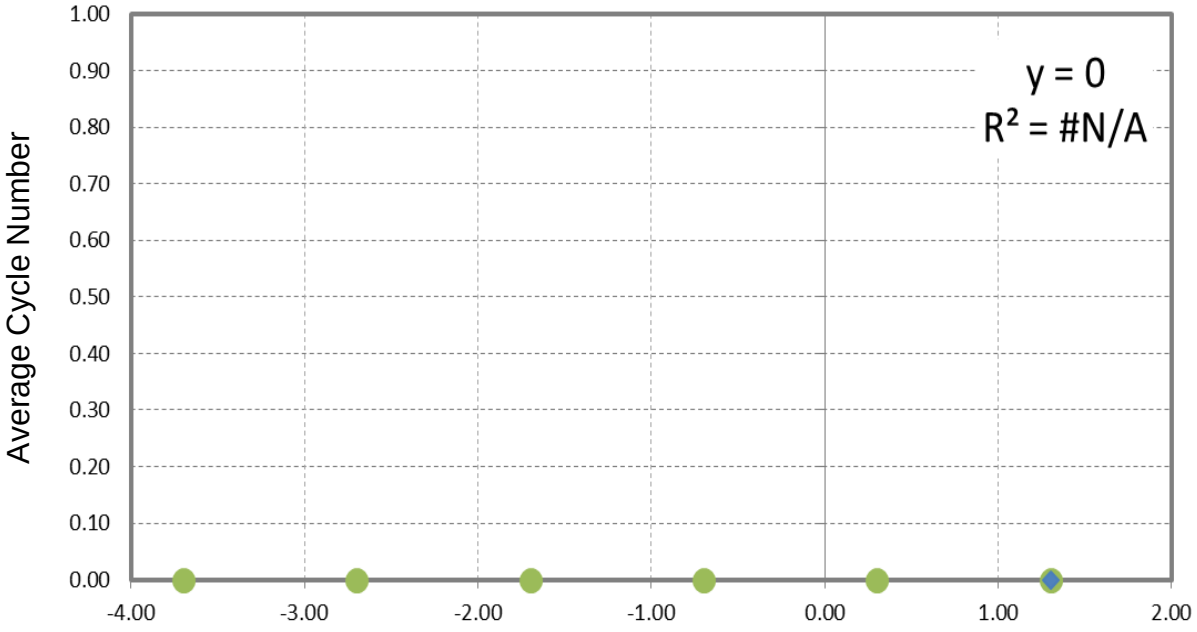


Diagram 10.29 QPCR graph

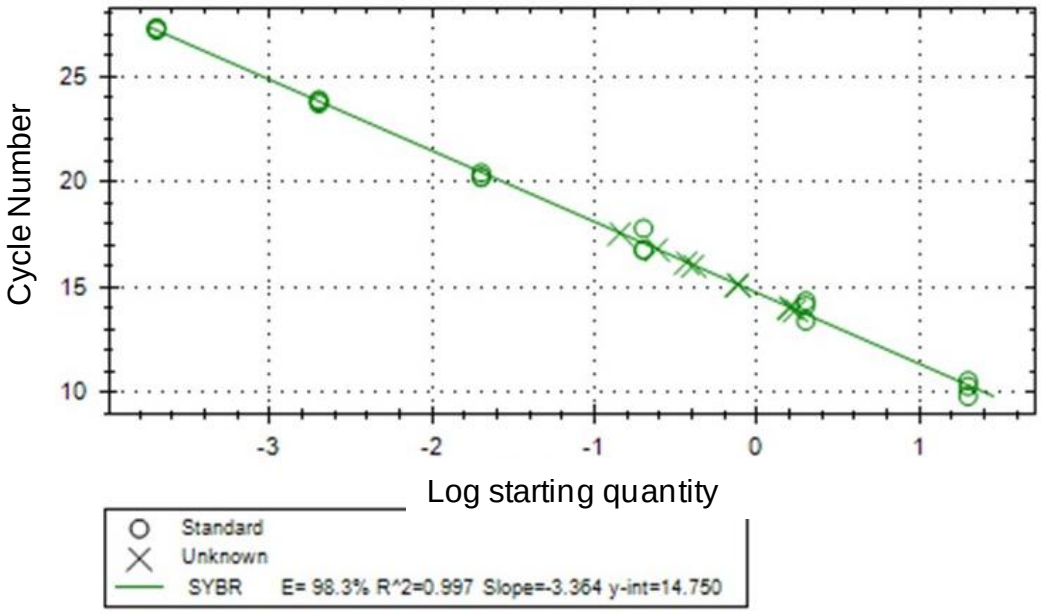


Diagram 10.30 Standard curve of QPCR as a benchmark
(Each cycle is the fluorescence first rises above the threshold level)

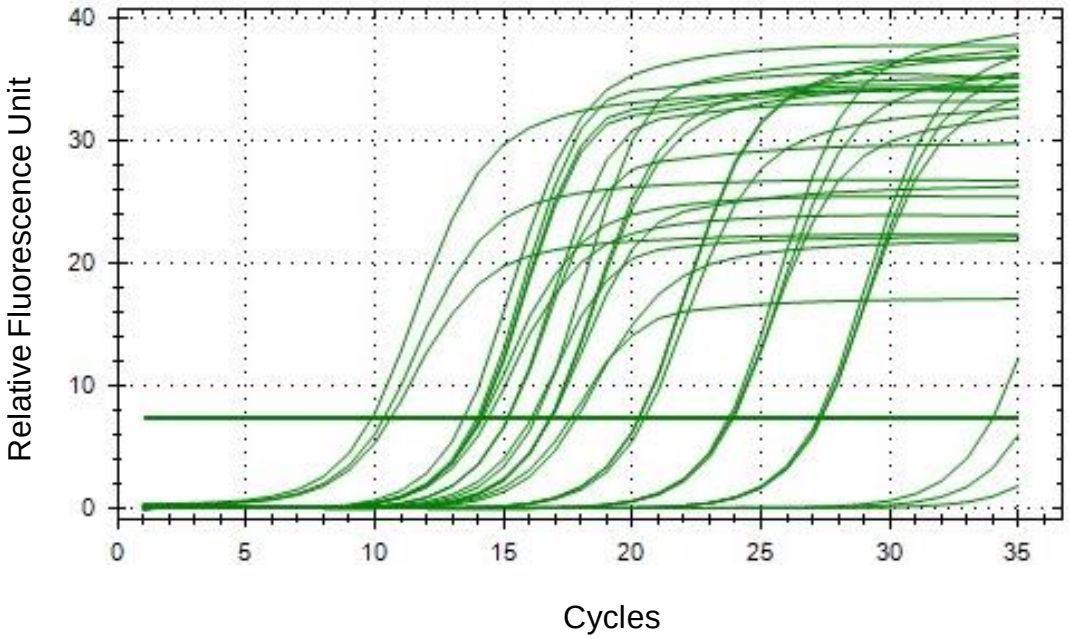


Diagram 10.31 The graph of QPCR cycles (Amplification Through Time)

Tables 10.34 (a-b) Qubit (Pooling)

Table 10.34a. Qubit (Pooling)

1	3.730	0.918	2.350	2.350	9.600	242.000	141.000	366.000	37.300	38.100	0.354	36.600
2	5.850	7.970	0.989	0.509	3.130	40.700	193.000	108.000	73.500	51.000	191.000	55.000
3	13.300	11.300	49.600	1.770	48.800	49.900	47.900	54.000	319.000	55.000	0.797	55.000
4	36.900	59.000	6.030	2.940	44.100	205.000	260.000	206.000	57.000	46.600	44.400	43.300
5	6.360	36.400	56.000	5.490	414.000	42.800	78.300	55.000	45.800	42.500	48.200	41.400
6	48.100	55.000	1.700	59.000	352.000	53.000	53.000	60.000	202.000	44.400	3.720	53.000
7	35.700	3.770	8.990	6.030	57.000	379.000	40.300	44.000	1.500	0.415	36.100	49.400
8	0.355	47.500	40.300	19.900	31.100	374.000	59.000	47.600	28.500	18.700	57.000	53.000
	nM conc: use my attached calculator to convert ng/ul to nM based on 600 bp size											
1	9.564	2.354	6.026	6.026	24.615	620.513	361.538	938.462	95.641	97.692	0.908	93.846
2	15.000	20.436	2.536	1.305	8.026	104.359	494.872	276.923	188.462	130.769	489.744	141.026
3	34.103	28.974	127.179	4.538	125.128	127.949	122.821	138.462	817.949	141.026	2.044	141.026
4	94.615	151.282	15.462	7.538	113.077	525.641	666.667	528.205	146.154	119.487	113.846	111.026
5	16.308	93.333	143.590	14.077	1061.538	109.744	200.769	141.026	117.436	108.974	123.590	106.154
6	123.333	141.026	4.359	151.282	902.564	135.897	135.897	153.846	517.949	113.846	9.538	135.897
7	91.538	9.667	23.051	15.462	146.154	971.795	103.333	112.821	3.846	1.064	92.564	126.667
8	0.910	121.795	103.333	51.026	79.744	958.974	151.282	122.051	73.077	47.949	146.154	135.897
	Then, add each library equimolarly (uL): To make 4800uL of 4 nM pool library, you need to add each of the below											
1	20.912	84.967	33.191	33.191	8.125	0.322	0.553	0.213	2.091	2.047	220.339	2.131
2	13.333	9.787	78.868	153.242	24.920	1.916	0.404	0.722	1.061	1.529	0.408	1.418
3	5.865	6.903	1.573	44.068	1.598	1.563	1.628	1.444	0.245	1.418	97.857	1.418
4	2.114	1.322	12.935	26.531	1.769	0.380	0.300	0.379	1.368	1.674	1.757	1.801
5	12.264	2.148	1.393	14.208	0.188	1.822	0.996	1.418	1.703	1.835	1.618	1.884
6	1.622	1.418	45.882	1.322	0.222	1.472	1.472	1.300	0.386	1.757	20.968	1.472
7	2.185	20.680	8.676	12.935	1.368	0.206	1.935	1.773	52.000	187.952	2.161	1.579
8	219.718	1.642	1.935	3.920	2.508	0.209	1.322	1.639	2.737	4.171	1.368	1.472
sum	1570.479											
	However, some library is too diluted (hilighted in blue above) and you only have 20 uL eluate max for each library											
1	20.000	20.000	20.000	20.000	8.125	0.322	0.553	0.213	2.091	2.047	20.000	2.131
2	13.333	9.787	20.000	20.000	20.000	1.916	0.404	0.722	1.061	1.529	0.408	1.418
3	5.865	6.903	1.573	20.000	1.598	1.563	1.628	1.444	0.245	1.418	20.000	1.418
4	2.114	1.322	12.935	20.000	1.769	0.380	0.300	0.379	1.368	1.674	1.757	1.801
5	12.264	2.148	1.393	14.208	0.188	1.822	0.996	1.418	1.703	1.835	1.618	1.884
6	1.622	1.418	20.000	1.322	0.222	1.472	1.472	1.300	0.386	1.757	20.000	1.472
7	2.185	20.000	8.676	12.935	1.368	0.206	1.935	1.773	20.000	20.000	2.161	1.579
8	20.000	1.642	1.935	3.920	2.508	0.209	1.322	1.639	2.737	4.171	1.368	1.472
sum	545.174											
	Pool the above and add (4800-545.174)=4255 uL buffer to make 4800 uL of 4 nM pooled library											

Table 10.34b. Qubit (Pooling the library)

1	3.730	0.918	2.350	2.350	9.600	242.000	141.000	366.000	37.300	38.100	0.354	36.600
2	5.850	7.970	0.989	0.509	3.130	40.700	193.000	108.000	73.500	51.000	191.000	55.000
3	13.300	11.300	49.600	1.770	48.800	49.900	47.900	54.000	319.000	55.000	0.797	55.000
4	36.900	59.000	6.030	2.940	44.100	205.000	260.000	206.000	57.000	46.600	44.400	43.300
5	6.360	36.400	56.000	5.490	414.000	42.800	78.300	55.000	45.800	42.500	48.200	41.400
6	48.100	55.000	1.700	59.000	352.000	53.000	53.000	60.000	202.000	44.400	3.720	53.000
7	35.700	3.770	8.990	6.030	57.000	379.000	40.300	44.000	1.500	0.415	36.100	49.400
8	0.355	47.500	40.300	19.900	31.100	374.000	59.000	47.600	28.500	18.700	57.000	53.000
	nM conc: use my attached calculator to convert ng/ul to nM based on 600 bp size											
1	9.564	2.354	6.026	6.026	24.615	620.513	361.538	938.462	95.641	97.692	0.908	93.846
2	15.000	20.436	2.536	1.305	8.026	104.359	494.872	276.923	188.462	130.769	489.744	141.026
3	34.103	28.974	127.179	4.538	125.128	127.949	122.821	138.462	817.949	141.026	2.044	141.026
4	94.615	151.282	15.462	7.538	113.077	525.641	666.667	528.205	146.154	119.487	113.846	111.026
5	16.308	93.333	143.590	14.077	1061.538	109.744	200.769	141.026	117.436	108.974	123.990	106.154
6	123.333	141.026	4.359	151.282	902.564	135.897	135.897	153.846	517.949	113.846	9.528	135.897
7	91.538	9.667	23.051	15.462	146.154	971.795	103.333	112.821	3.846	1.064	92.564	126.667
8	0.910	121.795	103.333	51.026	79.744	958.974	151.282	122.051	73.077	47.949	146.154	135.897
	Then, add each library equimolarly (uL): To make 4800uL of 10 nM pool library, you need to add each of the below											
1	52.279	212.412	82.979	82.979	20.313	0.806	1.383	0.533	5.228	5.118	530.847	5.328
2	33.333	24.467	197.169	383.104	62.300	4.791	1.010	1.806	2.653	3.824	1.021	3.545
3	14.662	17.257	3.931	110.169	3.996	3.908	4.071	3.611	0.611	3.545	244.668	3.545
4	5.285	3.305	32.338	66.327	4.422	0.951	0.750	0.947	3.421	4.185	4.392	4.503
5	30.660	5.357	3.492	35.519	0.471	4.556	2.490	3.545	4.258	4.388	4.046	4.710
6	4.054	3.545	114.706	3.305	0.554	3.679	3.679	3.250	0.965	4.392	52.419	3.679
7	5.462	51.724	21.691	32.338	3.421	0.515	4.839	4.432	130.000	469.880	5.402	3.947
8	549.296	4.105	4.839	9.799	6.270	0.521	3.305	4.097	6.842	10.428	3.421	3.679
sum	3926.198											
	However, some library is too diluted (hilighted in blue above) and you only have 20 ul eluate max for each library											
1	20.000	20.000	20.000	20.000	20.000	0.806	1.383	0.533	5.228	5.118	20.000	5.328
2	20.000	20.000	20.000	20.000	20.000	4.791	1.010	1.806	2.653	3.824	1.021	3.545
3	14.662	17.257	3.931	20.000	3.996	3.908	4.071	3.611	0.611	3.545	20.000	3.545
4	5.285	3.305	20.000	20.000	4.422	0.951	0.750	0.947	3.421	4.185	4.392	4.503
5	20.000	5.357	3.492	20.000	0.471	4.556	2.490	3.545	4.258	4.388	4.046	4.710
6	4.054	3.545	20.000	3.305	0.554	3.679	3.679	3.250	0.965	4.392	20.000	3.679
7	5.462	20.000	20.000	20.000	3.421	0.515	4.839	4.432	20.000	20.000	5.402	3.947
8	20.000	4.105	4.839	9.799	6.270	0.521	3.305	4.097	6.842	10.428	3.421	3.679
sum	782.275											
	Pool the above and add:											
	4017.725	uL buffer to make 4800uL of 10 nM pooled library										

Tables 10.35 (a-b) convert Ng to nM Calculator (Pooling)

Table 10.35a. Convert Ng to nM Calculator (Pooling)

Converting to nM			
STEP1	Insert your sample name here	GA765	
STEP2	Input the concentration of yous sample	3.2	ng/uL
STEP3	Insert the average length of your product	350	bp
STEP4	This is the concentration of your sample in nM	14.06593	nM
	Assuming a Mr of (Don't change this)	650	Da/bp
Doing your dilution			
STEP1	How many uL of 10 nM library do you want to make	130	uL
STEP2	Use this many uL of you concentrated stock	92.42	uL
STEP3	Use this much QIAGEN EB / 0.1% Tween20	37.58	uL

Table 10.35b. Convert Ng to nM Calculator (Pooling)

Converting to nM														
	Sample number	1	2	3	4	5	6	7	8	9	10	11	12	
STEP1	Insert your sample name here	GA1177	GA840	GA707	GA886	GA765	GA1041	GA1731	GA1750	GAJ0379	GAJ0165	GAJ0235	GAJ0212	
STEP2	Input the concentration of yous sample	22	20.8	43.8	16.8	26.3	31.1	12.5	27.5	14.6	23.2	23	17.9	ng/uL
STEP3	Insert the average length of your product	350	350	350	350	300	350	400	350	400	400	450	350	bp
STEP4	This is the concentration of your sample in nM	96.70	91.43	192.53	73.85	134.87	136.70	48.08	120.88	56.15	89.23	78.63	78.68	nM
	Assuming a Mr of (Don't change this)	650	650	650	650	650	650	650	650	650	650	650	650	Da/bp
Doing your dilution														
STEP1	How many uL of 10 nM library do you want to make	100	100	100	100	100	100	100	100	100	100	100	100	uL
STEP2	Use this many uL of you concentrated stock	10.34	10.94	5.19	13.54	7.41	7.32	20.80	8.27	17.81	11.21	12.72	12.71	uL
STEP3	Use this much QIAGEN EB / 0.1% Tween20	89.66	89.06	94.81	86.46	92.59	92.68	79.20	91.73	82.19	88.79	87.28	87.29	uL

Tables 10.36 (a-b-c) Dilution

Table 10.36a. Dilution details of library

Library concentrations and yields

Sample name	Concentration of undiluted library (pM)	Concentration of undiluted library (nM)	Concentration of undiluted library (ng/µL)	Library volume (µL)	Available amount of library (ng)
Homa Lib 1	4,759	4.76	2.06	25	51.5
Homa Lib 1	3,750	3.75	1.62	25	40.5
phiX	14,294	14.29	4.42	25	110.4

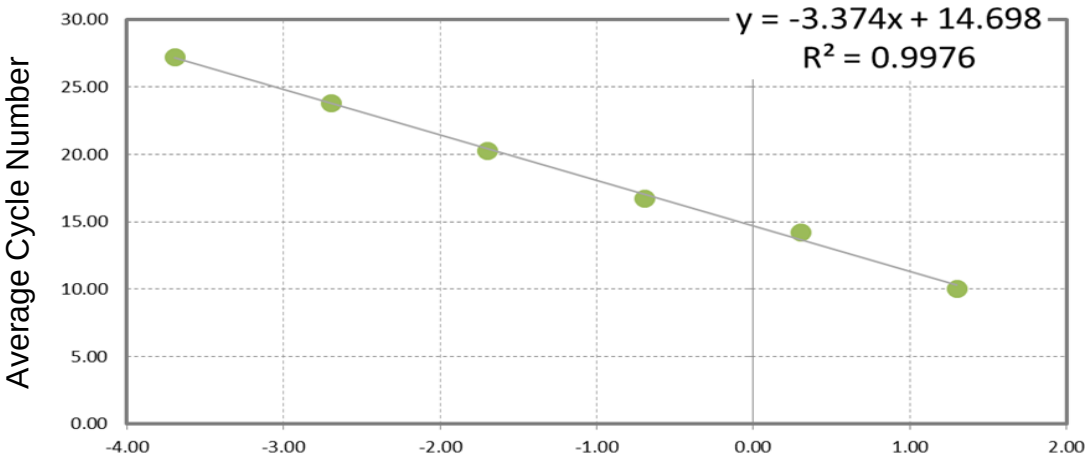
Table 10.36b. Dilution Summary of library

Library concentrations and yields

Sample name	Concentration of undiluted library (pM)	Concentration of undiluted library (nM)	Concentration of undiluted library (ng/µL)	Library volume (µL)	Available amount of library (ng)
Homa Library	3,971	3.97	1.84	25	46.0
pHiX	#DIV/0!	#DIV/0!	#DIV/0!	25	#DIV/0!

Table 10.36c. Dilution Analysis of library

Well	Std #	Conc (pM)	Cq	Outliers	Av Cq	Difference	Delta Cq
	1	20	10.31		10.08	0.24	-
	1	20		10.57		-10.08	
	1	20	9.84			-0.23	4.18
	2	2	14.36		14.25	0.11	
	2	2	14.14			-0.11	
	2	2		13.42		-14.25	2.53
	3	0.2	16.79		16.78	0.01	
	3	0.2		17.80		-16.78	
	3	0.2	16.77			-0.01	3.51
	4	0.02	20.22		20.29	-0.07	
	4	0.02	20.23			-0.06	
	4	0.02	20.43			0.14	3.51
	5	0.002	23.81		23.81	0.00	
	5	0.002	23.90			0.09	
	5	0.002	23.71			-0.10	3.45
	6	0.0002	27.18		27.26	-0.08	
	6	0.0002	27.31			0.05	
	6	0.0002	27.28			0.02	6.73
	NTC	-	33.99		33.99		
	NTC	-	n/a				
	NTC	-	n/a				



Log (concentration in per minute)

Diagram 10.32 Dilution graph (line chart)

Table 10.37 Quantification CQ result (Illumina)

File Name	Homa 11.04.2018.pcrd
Created By User	admin
Notes	Quantification Library Standard Curve
ID	
Run Started	04/11/2018 04:22:10 UTC
Run Ended	04/11/2018 06:18:06 UTC
Sample Vol	20
Lid Temp	105
Protocol File Name	Illumina Quant.prcl
Plate Setup File Name	Illumina Quant.pltd
Base Serial Number	CT012162
Optical Head Serial Number	785BR10746
CFX Manager Version	3.1.1517.0823.

10.11 Sequencing Steps

Among NGS methods, Illumina MiSeq sequencing is the most operative and widely used technology globally due to its low rate of error and the lowest cost per million bases, but requires short diagnostic regions of 300 base pairs to be effective (Table 39.) (Figures 9, 10.).

Table 10.38 Sample Sheet prepared for MiSeq run

Investigator Name	Homa								
Experiment Name	Homa								
Date	1/05/2018								
Workflow	GenerateFASTQ								
Application	FASTQ Only								
Assay	Nextera XT v2 Set A								
Description	Homa								
Chemistry	Amplicon								
[Reads]									
301									
301									
[Settings]									
ReverseComplement	0								
Adapter	CTGTCTCTTATACACATCT								
[Data]									
Sample_ID	Sample_Na	Sample_P	Sample_W	I7_Index_	index	I5_Index_	index2	Sample_P	Description
269073	269073			N701	TAAGGCG	S502	CTCTCTAT		
2335174	2335174			N702	CGTACTA	S502	CTCTCTAT		
2358340	2358340			N703	AGGCAGA	S502	CTCTCTAT		
2358338	2358338			N704	TCCTGAG	S502	CTCTCTAT		
2025255	2025255			N705	GGACTCC	S502	CTCTCTAT		
2370168	2370168			N706	TAGGCAT	S502	CTCTCTAT		
2362271	2362271			N707	CTCTCTAC	S502	CTCTCTAT		
2341799	2341799			N710	CGAGGCT	S502	CTCTCTAT		
2359120	2359120			N711	AAGAGGC	S502	CTCTCTAT		
2317408	2317408			N712	GTAGAGG	S502	CTCTCTAT		
1052548	1052548			N714	GCTCATG	S502	CTCTCTAT		
2363831	2363831			N715	ATCTCAG	S502	CTCTCTAT		
2335167	2335167			N701	TAAGGCG	S503	TATCCTCT		
2104350	2104350			N702	CGTACTA	S503	TATCCTCT		
2316761	2316761			N703	AGGCAGA	S503	TATCCTCT		
2363659	2363659			N704	TCCTGAG	S503	TATCCTCT		
2362243	2362243			N705	GGACTCC	S503	TATCCTCT		
2246934	2246934			N706	TAGGCAT	S503	TATCCTCT		
2397803	2397803			N707	CTCTCTAC	S503	TATCCTCT		
2039051	2039051			N710	CGAGGCT	S503	TATCCTCT		
2336387	2336387			N711	AAGAGGC	S503	TATCCTCT		
1061291	1061291			N712	GTAGAGG	S503	TATCCTCT		
2091051	2091051			N714	GCTCATG	S503	TATCCTCT		
2292425	2292425			N715	ATCTCAG	S503	TATCCTCT		
2317471	2317471			N701	TAAGGCG	S505	GTAAGGAG		
1061315	1061315			N702	CGTACTA	S505	GTAAGGAG		
2321893	2321893			N703	AGGCAGA	S505	GTAAGGAG		
2054573	2054573			N704	TCCTGAG	S505	GTAAGGAG		
2103697	2103697			N705	GGACTCC	S505	GTAAGGAG		
2257957	2257957			N706	TAGGCAT	S505	GTAAGGAG		
2317392	2317392			N707	CTCTCTAC	S505	GTAAGGAG		
2305329	2305329			N710	CGAGGCT	S505	GTAAGGAG		
2369927	2369927			N711	AAGAGGC	S505	GTAAGGAG		
2353243	2353243			N712	GTAGAGG	S505	GTAAGGAG		
228365	228365			N714	GCTCATG	S505	GTAAGGAG		
2320533	2320533			N715	ATCTCAG	S505	GTAAGGAG		
2320554	2320554			N701	TAAGGCG	S506	ACTGCATA		
2341406	2341406			N702	CGTACTA	S506	ACTGCATA		
1055464	1055464			N703	AGGCAGA	S506	ACTGCATA		
2367138	2367138			N704	TCCTGAG	S506	ACTGCATA		
2367329	2367329			N705	GGACTCC	S506	ACTGCATA		
2069119	2069119			N706	TAGGCAT	S506	ACTGCATA		
2192209	2192209			N707	CTCTCTAC	S506	ACTGCATA		
2305258	2305258			N710	CGAGGCT	S506	ACTGCATA		
2364220	2364220			N711	AAGAGGC	S506	ACTGCATA		
2321147	2321147			N712	GTAGAGG	S506	ACTGCATA		
2095287	2095287			N714	GCTCATG	S506	ACTGCATA		
2335203	2335203			N715	ATCTCAG	S506	ACTGCATA		
2321029	2321029			N701	TAAGGCG	S507	AAGGAGTA		
2335646	2335646			N702	CGTACTA	S507	AAGGAGTA		
269071	269071			N703	AGGCAGA	S507	AAGGAGTA		
2367327	2367327			N704	TCCTGAG	S507	AAGGAGTA		
2397814	2397814			N705	GGACTCC	S507	AAGGAGTA		
2066942	2066942			N706	TAGGCAT	S507	AAGGAGTA		
1061294	1061294			N707	CTCTCTAC	S507	AAGGAGTA		
2104293	2104293			N710	CGAGGCT	S507	AAGGAGTA		
2031600	2031600			N711	AAGAGGC	S507	AAGGAGTA		
2300488	2300488			N712	GTAGAGG	S507	AAGGAGTA		
2353240	2353240			N714	GCTCATG	S507	AAGGAGTA		
2297154	2297154			N715	ATCTCAG	S507	AAGGAGTA		
2314629	2314629			N701	TAAGGCG	S508	CTAAGCCT		
2300763	2300763			N702	CGTACTA	S508	CTAAGCCT		
269072	269072			N703	AGGCAGA	S508	CTAAGCCT		
2103829	2103829			N704	TCCTGAG	S508	CTAAGCCT		
2257903	2257903			N705	GGACTCC	S508	CTAAGCCT		
2053822	2053822			N706	TAGGCAT	S508	CTAAGCCT		
2317499	2317499			N707	CTCTCTAC	S508	CTAAGCCT		
2151410	2151410			N710	CGAGGCT	S508	CTAAGCCT		
2032789	2032789			N711	AAGAGGC	S508	CTAAGCCT		
2341629	2341629			N712	GTAGAGG	S508	CTAAGCCT		
2247018	2247018			N714	GCTCATG	S508	CTAAGCCT		
2369953	2369953			N715	ATCTCAG	S508	CTAAGCCT		
2031497	2031497			N701	TAAGGCG	S510	CGTCTAAT		
269070	269070			N702	CGTACTA	S510	CGTCTAAT		
2231581	2231581			N703	AGGCAGA	S510	CGTCTAAT		
2112647	2112647			N704	TCCTGAG	S510	CGTCTAAT		
2236445	2236445			N705	GGACTCC	S510	CGTCTAAT		
2305193	2305193			N706	TAGGCAT	S510	CGTCTAAT		
2317500	2317500			N707	CTCTCTAC	S510	CGTCTAAT		
2264832	2264832			N710	CGAGGCT	S510	CGTCTAAT		
2031498	2031498			N711	AAGAGGC	S510	CGTCTAAT		
1052547	1052547			N712	GTAGAGG	S510	CGTCTAAT		
2025140	2025140			N714	GCTCATG	S510	CGTCTAAT		
2400352	2400352			N715	ATCTCAG	S510	CGTCTAAT		
2321061	2321061			N701	TAAGGCG	S511	TCTCTCCG		
2103668	2103668			N702	CGTACTA	S511	TCTCTCCG		
228403	228403			N703	AGGCAGA	S511	TCTCTCCG		
2322085	2322085			N704	TCCTGAG	S511	TCTCTCCG		
2358337	2358337			N705	GGACTCC	S511	TCTCTCCG		
2305339	2305339			N706	TAGGCAT	S511	TCTCTCCG		
2305213	2305213			N707	CTCTCTAC	S511	TCTCTCCG		
2317863	2317863			N710	CGAGGCT	S511	TCTCTCCG		
2121998	2121998			N711	AAGAGGC	S511	TCTCTCCG		
2247029	2247029			N712	GTAGAGG	S511	TCTCTCCG		
2381037	2381037			N714	GCTCATG	S511	TCTCTCCG		
2192210	2192210			N715	ATCTCAG	S511	TCTCTCCG		

Figures 10.9 (a-b-c) Sequencing run (MiSeq)

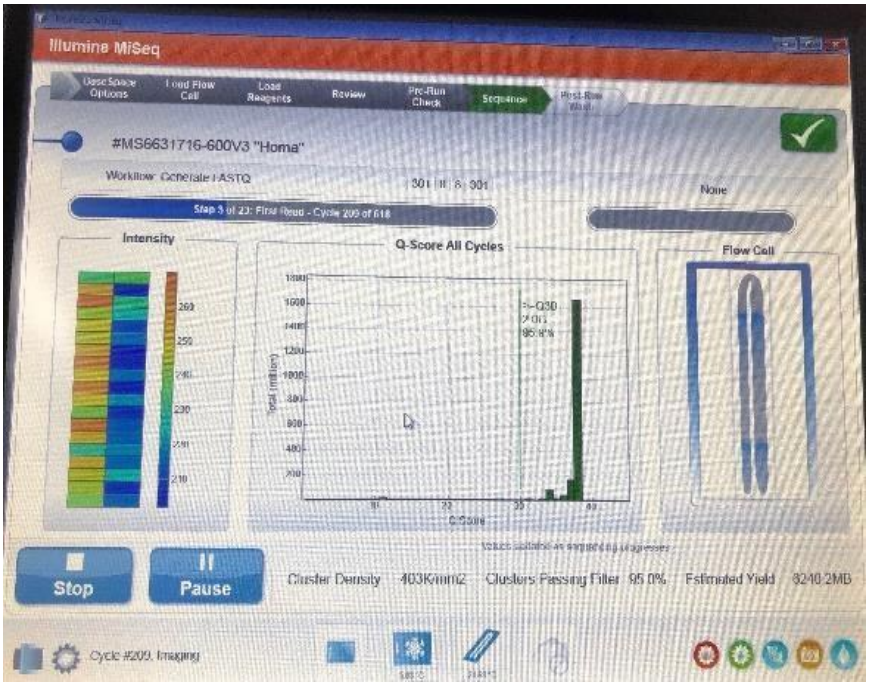


Figure 10.9a. Sequencing run (starting phase)

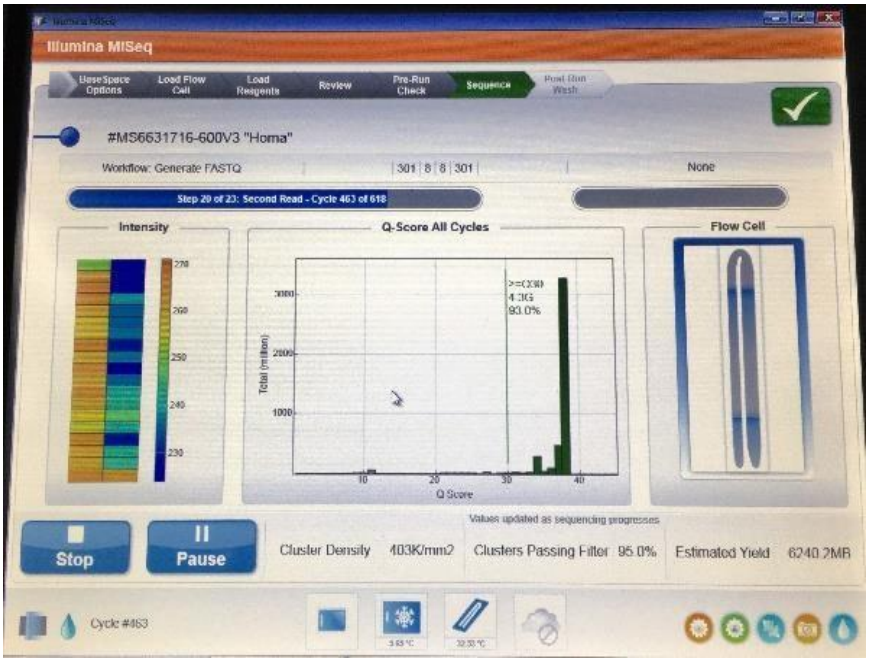


Figure 10.9b. Sequencing run

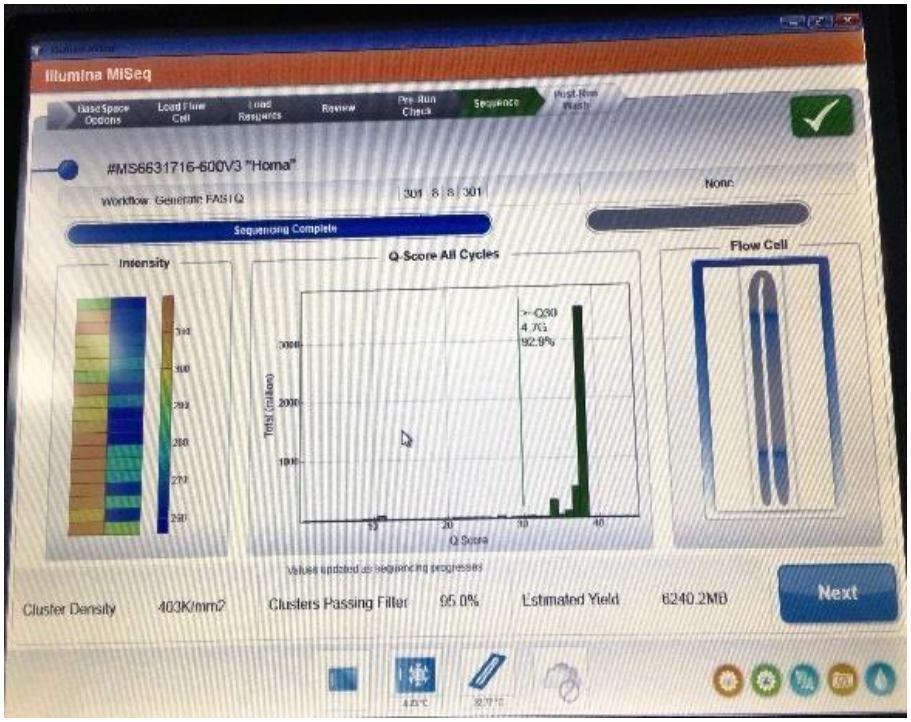


Figure 10.9c. Sequencing run

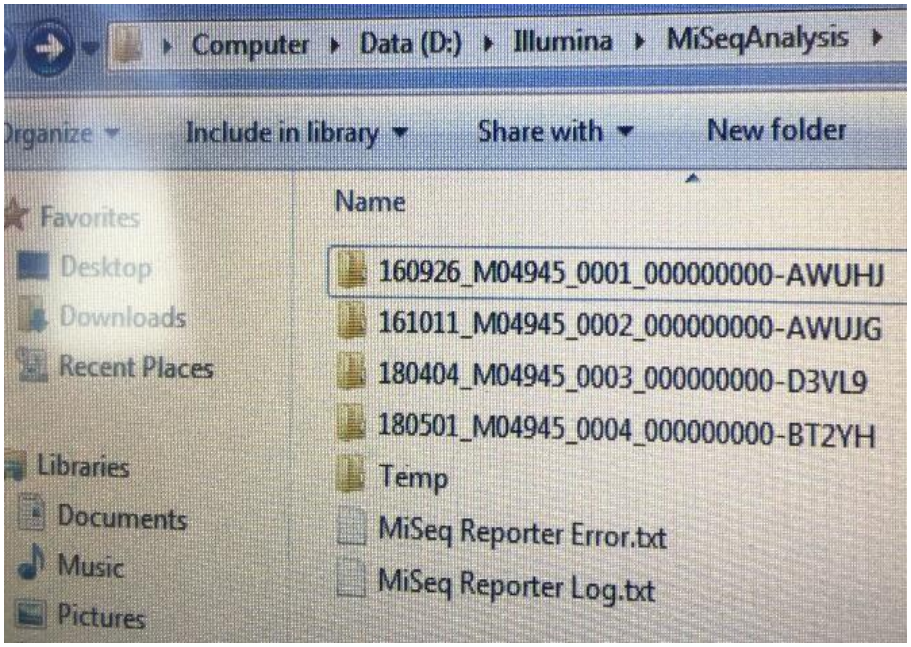


Figure 10.10. Sequencing results (folders space)

Complete details of methodology for 96 *Psilocybe* Melbourne herbarium samples

Table 10.39 Sample.xlsx

It is difficult to identify the species of the fungi based solely on morphological observation. Modern identification methods built on a molecular analysis are gaining popularity with some authors considering molecular identification essential (Maruyama et al. 2003). In addition, molecular genetics is used to assist in the differentiation of species. The identification of fungi by means of a DNA-based test, which can be used in combination with morphological features, assist us to be assured of our identification (Lee, Cole & Linacre 2000a). A DNA-based identification technique allows the identification of samples when there is a lack of morphological features such as in the mycelium or spore stage (Nugent & Saville 2004).

11 Result / Data Analysis (Phylogenetic analysis, Morphological analysis, Molecular analysis)

Based on the data from morphology observation it can be said that there are a few different clusters from different part measurements. The data shows that main character, which is spore, can play meaningful role in determining the neighbouring relationship. As it obtains from diagram 10, it can be inferred that founded on spore diameters there are mainly seven clusters of different characteristics among 96 samples. This is almost the same as results from sequence data. It shows that there is a correlation between morphology and molecular data.which demonstrates the alignment of our mixed data.

The use of the ITS region for molecular identification of fungi goes back to the early 1990s (Hibbett, DS et al. 2011). The region is composed of the two highly variable spacers ITS1 and ITS2 which, jointly or separately, are often species specific, and the intercalary, much conserved 5.8S gene. The sequence conservation in the proximate genes, coupled with numerous copies of the ribosomal operon, makes primer design and PCR amplification of the ITS region straightforward even from low-DNA quantity substrates such as old herbarium specimens. Clone libraries vs direct sequencing and pyrosequencing vs Sanger sequencing are the subject of our argument (Hibbett, DS et al. 2011). It would be worthy, if we could sequence roughly a handful of previously un-sequenced fungal type specimens / ex-type cultures in the herbarium / culture collection and make those sequences openly available (Nilsson, Abarenkov & Kõljalg 2016). ITS DNA sequences created in fungal ecology revisions utilize NGS. This tool is planned for use with 454 pyrosequencing and is likewise applicable for use with any sequencing policy that agrees for BLAST searches alongside the specified ITS database. For each ITS, SSU, LSU and EF sequence the BLAST production grade, all topmost hits at the rank of species with a tying E-value, that matches the exact sequence (Dannemiller et al. 2014). The best contigs (final assembled sequences called contigs) with dimension identical the predictable sequences regions can be directly used for ITS based species identification at the NCBI Nucleotide BLAST or the ISHAM ITS database (Ahmed 2016).

UNITE reflects the fungal ITS sequences in the INSD, GenBank, EMBL and DDBJ (Kõljalg et al. 2013). Parsimonious informative give satisfactory amplification and band resolution. Since parsimony analysis might not be highly informative, because of the large number of conserved sites, an alternative algorithm for phylogenetic analysis, maximum likelihood, should be employed. Maximum likelihood approach, which is the preferred method for DNA, is based on studying trait evolution, use substitution (transition) probabilities. FHiTHINGS (Fungal High Throughput Identification) offers a simple high throughput software answer for analysing and sorting BLASTn results from ITS based NGS studies (Dannemiller et al. 2014).

Several polymerase chain reaction (PCR) based methods of DNA analysis have been employed in species identification, e.g., random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and real-time PCR and DNA sequencing. Some of these methods, such as RAPD and AFLP methods, analyse the whole genome and are applied in the identification of polymorphisms and determination of genetic distance between species, but are not suitable for identification of closely-related species. Other methods, e.g., real-time PCR and DNA sequencing, are based on the amplification of DNA fragments of taxonomic relevance. Apposite projects to families and genera can be qualitatively assessed, using UPGMA dendrogram and Nonmetric MultiDimensional Scaling (NMDS) ordinations based on a matrix of pairwise sequence distances designed through the full dataset (Osmundson et al. 2013).

In order to set up a reliable profile database, it is significant to first approve by ITS sequencing the species of the samples being tested. Although these ‘sequence-based classification and identification’ methods are a powerful means to rapidly detect hidden diversity, careful interpretation of these data is needed to make accurate inferences. It is time-consuming and requires expertise. My study works on the isolation and complete sequencing of three regions of the herbarium specimens. The whole data are collected from experiments in the laboratory. Samples are powdered by mortar and pestle. The powder then is centrifuged, incubated and different buffers and various solutions are added. Sequence data PCR products are separated on Agarose gel and visualised by

UV light using Gel imagine software (Lee, Cole & Linacre 2000a). Quantification of library is double-checked with QPCR, as it has been checked once by Bioanalyser and showed the same accuracy.

Each sample are compared to all other samples on the base of matched bands (Linacre, A, Cole & Lee 2002). The empirical data is the internal transcribed spacer (ITS) region of nuclear ribosomal DNA from fungi, the formal barcoding region in this kingdom. Fungi have many copies of the rDNA gene that sorts the locus acquiescent to PCR (Lee, Cole & Linacre 2000a). Amongst the six primers, ITS delivered more reliable data for identification purposes, by grouping samples of the similar species and clustering thoroughly correlated species together in a dendrogram based on amplicon similarities. In terms of sequence variation, success rate of each marker, PCR success rate, and sequencing success rate ITS showed the most reliable results.

Through the last couple of decades, a rising number of studies use sequence clusters as units for taxonomic diversity (Nugent & Saville 2004). Considering molecular techniques are fast and more accessible. Ideally, all barcode sequences contained in either database should have been derived from a vouchered specimen and the most reliable results and the final alignments used for computing trees. Amplification efficiencies of 3 primer pairs targeting 3 genetic markers are tested across 96 species. The phylogenetic analyses are well resolved the problem of classification this taxa by support of these amplified regions and showed the satisfactory level of neighbouring. The annotated processing of fungal DNA sequence data of ITS region should contain the following; 1) elimination of primer sequences adaptors and multiplex tags, 2) quality trimming built on sequencing quality scores and length, 3) high throughput comparison of recovered sequences with an ITS sequence databases, 4) the capability to select taxonomically categorize and quantify the sequence abundance of database search outcomes database.

Nucleotide sequences are aligned and trees are assembled utilizing the neighbour joining distance algorithm (Phenetics=Taximetrics genetic method) for the ITS1 and the 5 protein of the nLSU rRNA. Comparable trees are generated using Parsimony analysis yet the N-J algorithm has the benefit of being fewer

computationally severe and using a base-change model that adopts adaptable degrees of nucleotide swap (Nugent & Saville 2004).

Sequencing library preparation workflow is getting much easier, thanks to the pioneering studies. Simple and rapid library preparation protocols for DNA sequencing, still data analysis remains the most interesting stage in this magnificent technology (Ahmed 2016). Despite the increasing number of barcoding sequences in the database, taxon sampling is still mainly absent and the majority of the sequences still lack descriptive species.

The special authoritative standing of type specimens in systematics equally gives rise to the need to redesignate typical sequences on an ordered foundation. Not all sequences from type specimens form perfect reference sequences though. Type specimens in contrast might be tens to hundred years old, making it challenging to acquire long high-quality DNA sequences (Kõljalg et al. 2013).

When DNA data from the type specimens become available, we can have a direct bond between species names and sequence data. The attained sequences are edited and assembled along with the reference sequence from the type specimen quality (in terms of degrees of fragmentation) (Hosaka, K & Uno 2013). Due to the shortage of high-quality type sequences, we looked to designate a sequence that initiated from the similar country or geographical zones as the type specimen sequences from vouchered fruiting bodies. Living cultures are preferred over un-cloned sequences from other bases (Kõljalg et al. 2013). All sequences are aligned using Multiple Alignment by Geneious software (Kaewgrajang et al. 2020).

In addition to morphological studies, molecular systematic analysis of the genus is critical. Clearly development in traditional styles joined with other biochemical / serological methods and combination of various molecular practices (DNAbased) has delivered new data on these aspects but, for a clearer picture and a better understanding, a combination of all attitudes (polyphasic) is crucial. There is an essential to unravel the taxonomic diversity of species groups (Jeewon & Hyde 2007). The reference sequences are deposited (http://unite.ut.ee/repository.php) for usage by the technical community for example local sequence match searches and in the QIME Pipeline. The structure and the statistics are updated

regularly as the number of public fungal ITS sequences increase (Kõljalg et al. 2013).

Molecular data has delivered clarification of the phylogenetic relationships among the species, and it has been possible to measure the taxonomic value of the morphological characters used in the genus. Moreover, molecular data provides invaluable information to support morphological data and support the identification. Integrating macro- and micro- morphological characters as well as molecular phylogenetic analysis are important to well characterize a species within this genus. For barcode gap analysis, the sequences are aligned using MUSCLE with system parameters (Kakoti et al. 2021).

Prior to construction of phylogenetic tree, total nucleotide length (bp) and variable sites are calculated using Geneious software (Tamura et al. 2013). Phylogenetic tree is constructed using two different methods; Maximum Likelihood (ML) and Maximum Parsimony (MP). To test branch support, bootstrap analysis is used with 1000 replicates. Most parsimonious trees proposing the molecular variances related to geographical distributions in this species, are comparatively unimportant (Zhang et al. 2010).

The alignment results are adjusted manually where necessary. Gaps are treated as missing data. All positions containing gaps and missing data are eliminated. Bootstraps are performed and the robustness of the different branches of interest is confirmed by significant bootstrap values (> 80%). The topologies of the phylogenetic trees produced by distance matrix and parsimony criterions based on this data set agreed with that of the maximum likelihood. Maximum likelihood analyses relied on models determined as best-fits to each data set by comparing diverse nested copies of DNA substitution in a hierarchical hypothesis-testing outline, resulting the probability proportion test as applied by MODELTEST. Conversely manual arranging may be fit for hundreds of sequences, it is difficult once the number of general sequences exceeds to hundreds of thousands, which is normal for NGS ecology studies database (Dannemiller et al. 2014).

In case of lots of variety in loci, it can be the result of contamination, wrong loci selection, heterozygosity or paralogous. The factors considered for analysis of the sequence data are heterogeneity, identity, variability and heterozygosity. The

mean intra-specific divergence (%), maximum intra-specific divergence (%) and minimum inter- specific divergence (%) of each species to the nearest species are presented in the dendrogram tree. Further analysis based on multiple barcode loci and combinations of those pieces of information with morphological features of the subgroups will provide a more detailed scenario of the evolutionary relationships and taxonomic positions as different species or subspecies (Kakoti et al. 2021).

For the purpose of species identification, the obtained sequences are compared with sequences available in the National Centre for Biotechnology Information (NCBI) and internet database (GenBank), using the BLAST program.

Comparative sequence analysis is appeared in almost all arenas of biological sciences. A short-standardized sequence that can differentiate the individual from the species, forms the basis of DNA barcoding. Barcoding is typically carried out by the retrieval of a DNA sequence i.e., a barcode from a specific gene region. This unknown barcode is then compared with the other barcodes present in the library of reference barcode sequences (Rai et al. 2014). Sequence comparison of the ITS region is commonly used in taxonomy and molecular phylogeny relationships. Sequences are inspected using equally weighted maximum parsimony (MP), maximum likelihood (ML), differentially weighted maximum parsimony (WP) and distance methods (NJ). Transitions and transversions are assessed using maximum likelihood. Intron sequences are examined using maximum parsimony distinctly and in arrangement with exon sequences. A huge number of indels and countless overlapping, happened in the intron alignment that contain distantly associated taxa. These indels are recorded as missing. After elimination of unclearly aligned regions in the data set, these parsimony informative, are identified and rescored as separate presence / absence characters (Chi-square tests of homogeneity of base frequencies crosswise taxa produce non-significant outcomes in PAUP* under the ‘‘Base Frequencies’’ option) (Harmon 2005), unlike comparisons between distantly correlated taxa where nucleotide configuration bias (Foster & Hickey 1999) or sequences of paralogous loci among closely related taxa (Matheny et al. 2002).

There are about 100,000 species of fungi around the world (Hibbett, DS et al. 2011). Molecular genetic data nowadays play a noticeable title role in delimiting fungal species and understanding evolutionary associations in lichen. Molecular data are thus mainly valued for evaluating traditional species boundaries and for species delimitation in overall (Nugent & Saville 2004).

Molecular analyses are very helpful in determining the relationship in closely related species. Analysis of molecular data combined with morphological data resolves many disputes, not resolved by morphology alone. The barcoding results of some of our specimens are also confusing, because their sequences matched with several species from the database with equal percentage of identities. PCR amplification of DNA extracted from environmental samples with diverse and unknown sequences and unknown concentrations may be exposed to several kinds of bias. After the removal of primer sequences and ambiguous bases, chimeric and low-quality sequences, DNA alignments are checked by eye, and ambiguously aligned positions are manually adjusted. Phylogenetic tree constructed using the neighbour joining distance algorithm and analysis is based on the aligned sequences from the three locus. Numbers indicate percentage bootstrap support (100 replications) where values greater than 50% are indicated on the branches. Unidentified samples used as an assessment of our investigational approach. A taxon name specifies the isolate encloses the controlled elements (Nugent & Saville 2004).

During the quality-filtering step, sequences that are too short or too long (< 200 bp or > 1000 bp), those with homopolymer runs longer than eight nucleotides or with a mean quality score of < 25 are removed. Bootstrap ML values more than 70% and PPs above 0.95 are considered strong evidence for robust support. Comparison with GenBank sequences enable identification of all 96 samples at least at the genus level. The starting tree for branch swapping in the ML analyses is the tree with the highest ML score among nearest neighbour interchanges (NNI). Several computationally faster alternative actions for calculating bootstrap and jack-knife values are also employed in PAUP*, including "fast" bootstrapping and "fastjac" jack-knifing, which generate very similar topologies to those of the more optimized and more computationally intensive search schemes, albeit with slightly lower support ideals for most nodes (Moncalvo et al. 2000).

Trees derived from defined sequences reveal enlarged resolution, greater assurance for some relationships, and comparatively uniform terminal branch lengths (Matheny et al. 2002). Initial tree(s) for the heuristic search are obtained automatically by applying Neighbour-Join and Bio NJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach and then choosing the topology with superior log likelihood rate. The broad approval of DNA barcoding trusts on public repositories such as the INSDC (http://www.insdc.org/), which accessions hundreds of thousands of sequence entries (Vilgalys & Hester 1990).

Maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference are used to estimate phylogenetic relationships among investigated fungi using individual gene datasets and the combined dataset in order to assess within- vs. among-species nucleotide variation (‘‘barcode gaps’’). Barcode gap and multi-dimensional scaling analyses reveal that some of the tested candidate markers have universal properties providing adequate infra- and inter-specific variation that make them attractive barcodes for species identification. However, based on barcode gap analyses, we identified genomic markers that have a superior identification performance than the others and genomic markers that are not indicated for the identification of some genera. In compare to simple sequence likeness thresholds OUT-picking for delineating presumed evolutionary meaningful units, the Automatic Barcode Gap Discovery (ABGD) technique is a mechanical route that sorts sequences into hypothetical species centred on the presence of a barcode gap observed, when intraspecific genetic distances are less than those amongst organisms from diverse species. In communal the ABGD, GMYC and PTP analytical protocols using single-locus data are repeatable and computationally relatively fast providing a treasured start point for a prime outlook into species boundaries in understudied groups that can be approved with subsequent studies. Similarly, analysis of single-locus data can be used to authenticate traditional phenotype-based species boundaries and sort candidate species that have previously been unidentified within nominal species. Analysis of single-locus datasets would like fail to properly delineate species. In associate to single-locus and strictly phenotype based lines for species delineation, analysis of genetic archives collected from free genomic regions can

deliver robust norms of species boundaries with increasing reassurance sequences from several independent loci. It offers a vital source of data for species delimitation studies plus just advanced copies that pool separate gene genealogies and species phylogenies thru forming the coalescent history of markers (Leavitt, Moreau & Thorsten Lumbsch 2015).

The fact that, there is much variation in ITS, that facilitates to differentiate even closely associated species (Hussein et al. 2014). The nuclear ribosomal internal transcribed spacer (ITS) region is the primary choice for molecular identification of fungi. Its two highly variable spacers (ITS1 and ITS2) are usually species specific, whereas the intercalary 5.8S gene is highly conserved. For sequence clustering and BLAST searches, it is often advantageous to rely on either one of the variable spacers but not the conserved 5.8S gene. To identify and extract ITS1 and ITS2 from large taxonomic and environmental data sets is, however, often difficult and many ITS sequences are incorrectly delimited in the public sequence databases. A perhaps lesser-known complication with the ITS region in the context of molecular identification lies in its composite nature. The neighbouring SSU (immediately upstream of ITS1) and LSU (immediately downstream of ITS2) genes are much conserved, as is the intercalary 5.8S gene. The ITS1 and ITS2 spacers, on the other hand, are very variable. To subject sequences featuring both variable and conserved parts to similarity searches such as BLAST in the International Nucleotide Sequence Databases does not always produce the intended or correct results from the perspective of species identification. The conserved sequence parts likely find a match in the databases regardless of whether or not the variable part does, and so the outcome of the BLAST search may be more dependent on the length of the conserved component than the information content in the variable one. This would not be a concern if the reference databases featured an exhaustive taxon sampling of sequences of comparable length. Unfortunately, ITS sequence data are available only for about 1-5% of the estimated 1-5 million species of fungi and the public fungal ITS sequences come in very different degrees of coverage of the region, cautioning against cursory – or fully automated – inspection of BLAST results. Around 11% of the 86, 000 BLAST searches undertaken, produce a different result (nonsynonymous species name) depending on whether the full ITS region,

or just the variable regions, is used in the search. Differentiating the individual components of the ITS region is not trivial. While SSU, 5.8S and LSU are conserved, they are regularly too variable for simple pattern matching approaches via regular expressions for their identification. To undertake it with data sets produced by high-throughput DNA sequencing techniques such as pyrosequencing– where the number of sequences may exceed hundreds of thousands – is intractable. The short sequences are used to query GenBank using BLAST directed to target taxa (fungi) and non-target taxa (Eukarya) (Asemaninejad et al. 2016).

The UNIX software package – Fungal ITS Extractor – automatically identify, annotate and extract ITS1 and ITS2 from fungal ITS sequences. The software centred on profile hidden Markov models (HMMs) computed from large, kingdom-wide alignments for the 3′ end of SSU, the 5′ and 3′ ends of 5.8S, and the 5′ end of LSU. Profile HMMs are statistical models to represent the position specific variations and dependencies typically observed in multiple sequence alignments; without having to store the full alignment, the HMMs are still able to account for the fact that a certain proportion of the sequences may contain, for example, a 'T' instead of an 'A' in some given position, while other positions appear invariable. All query sequences are filtered through the HMMs, and extractions are made according to which HMMs that produced significant matches. A second use of the software is to filter out non-ITS sequences from large sequence data sets. However, the Fungal ITS Extractor is not impeccable. In larger fungal ITS data sets heterogeneous taxonomic coverage, the proportion of missed or incorrect extractions – although typically detected as such by the program – can approach 1% (Bengtsson‐Palme et al. 2013).

Microsatellite analysis is the common technique to study the genetic diversity between fungal isolates (Zoll et al. 2016). Species defined as a group of organisms involve series of effective interbreeding (Selosse, Vincenot & Öpik 2016). It is well known that such MOTUs do not basically link to species, but they are treated as such after evaluating diversity and trying copies (Ryberg 2015). Sequence data which are compared in Genetic distance Programme will be submitted in Gene Bank after submission (Nugent & Saville 2004) (Lee, Cole &

Linacre 2000b). The size of these three regions is analytic of the species. Dendrogram can be stablished by the genetic distance (GD) value or similarity matrix by using different software (Lee, Cole & Linacre 2000b) or using the neighbour joining distance algorithm (Nugent & Saville 2004). Phylogenetic tree is constructed from the nucleotide sequences of RNA (Maruyama et al. 2003). Phylogenetic relationship of the unknown and known species of *Psilocybe* taxa are obtained by GenBank (Tshivhandekano et al. 2014). The phylogram can be gained by trace characters from Bayesian (Ramírez-Cruz et al. 2013). Based on congruent molecular and morphological data, only sequences with complete nuclear ribosomal ITS from permanent collections, whose taxonomic identifications are curated by specialists (voucher specimens) and deposited in GenBank are used. Taxonomic information regarding the specimens is enriched, when available, from the UNITE database. This step is used after downloading sequences from GenBank and before logical and quality filters are applied. To determine the phylogenetic relationships, sequence analysis of the ITS regions of the rDNA repeats is performed and data compared to related species retrieved from GenBank. For each fungal isolate, sequences generated from the three regions together with a reference sequence obtained from GenBank are aligned using Geneious to obtain an assembled sequence. The species delimitation program, Bayesian phylogenetic and phytogeography are the tools for the analysis. Alignment is manually adjusted to allow maximum alignment and minimize gaps. Phylogenetic analyses are based on maximum parsimony (MP) performed in Geneiuos software. Clade stability is assessed by a bootstrap analysis, each with replicates of random stepwise addition of taxa recombination, mutation genetic and genomic data analysis. Furthermore, an exact sequence match with a known isolate in the database can be considered very close to an unambiguous species identification. Ambiguously aligned portions are removed manually. Diverse datasets and operational standards may give conflicting or puzzling results due to multiple evolutionary progression happening within and between populations. Enigmatic species level ancestries are usually recognized, using molecular facts.

Based on our NGS data in most cases it is probable to allocate a single consensus sequence as a reference DNA barcode for a singular sample.

Sequence information gained from comparable sequencing of each specimen through NGS is a peerless feature that cannot be practically reached through Sanger sequencing (Shokralla et al. 2014). It must be accepted that sequence data chiefly, those from single loci have limitations for taxonomy and phylogenetic restoration. To estimate the number of MOTUs indicated in the ITS sequences, we attained hierarchical clustering. Any sequence that failed to success is left as a singleton (Hibbett, DS et al. 2011).

DNA sequence data generated by Sanger sequencing technology characterized by justly limited size (+/- 800 bases single read). With the outline of next generation sequencing technologies, considerable sequence data with shifting grade of quality become reachable. One another issue on NGS data analysis is the infinite volume of data generated which is beyond the computing frame of most clinical set. In detail most of NGS platforms have some data analysis functionally which can be done on the same sequencing mechanism. Regardless of NGS platform used, sequence data normally deposited in script file in Fastq format which is the introductory material (Ahmed 2016).

The usage of NGS will obviously resolve the problem by providing access to the sequences of the dual alleles in the occasion of heterozygosity as NGS approaches sequence the DNA molecules individually, unlike sanger sequencing (Selosse, Vincenot & Öpik 2016) (Parisod & Broennimann 2016). Sanger sequences are logically assembled from two or more primer reads (Nilsson, Abarenkov & Kõljalg 2016). Low quality sequence reads and / or sequence contamination must to be disconnected from data sets before any subsequent data handling or analysis (Ahmed 2016). Any sequences covering undefined base calls are removed as well as any reads that did not readily match the primer sequence (Asemaninejad et al. 2016). Academics should make it a routine to trim such interfering parts prior to sequence submission (Nilsson, Abarenkov & Kõljalg 2016). The existence of chimeras, deeper paralogues, multicopy markers and pseudogenes are the key source of fault (Větrovský et al. 2016).

In deep sequencing, the full DNA content of biological model is sequenced. For trustworthy outcomes a depth or coverage of 10 to 15 is required, this means that each single nucleotide in the trial, is read at least 10 to 15 times (Zoll et al. 2016).

The demand of high-quality reference sequences has increased rapidly due to the increasing use of high throughput sequencing services. To generate a brief set of reference sequences, UNITE applies a double clustering procedure, first clustering all sequences to roughly the subgenus / genus level and then to closely the species level (Kõljalg et al. 2013).

Genetic diversity studied by three pairs of primers. ITS typically valuable at species level, LSU valuable for genus level but not for species level. If there is a comprehensive reference record over the organism studies, sequences can be accepted to species simply by finding the corresponding sequence in the database. However, while revising very diverse societies and / or organism groups, the reference databases are frequently far from complete (Ryberg 2015). Much like other sources of data, DNA sequences will not give rise to appropriate exact outcomes excepting they are generated and analysed in a right precise mode (Nilsson, Abarenkov & Kõljalg 2016).

Any operator with a freshly generated set of DNA sequences, should take steps to guarantee that, any presumed taxonomic associations of the sequences check out a BLAST search in UNITE or INSDC is naturally adequate to rule out contamination or sever cases of misidentification. Any sequence found to align well in the primary part of the alignment, but to align poorly in the following half merits additional scrutiny. A BLAST search by manual check of the results is alternate way with a comparable possible for Chimera innovation. Fortunately, BLAST supports reverse matching sequences by default and accounts for their nature alignment chapter. A strand flag of plus / minus or minus / plus in the BLAST output describes opposite read instructions of the request and the reference sequence. The operator can reorient converse paired sequences in alignment editors. DNA ambiguity codes such as N, R and S distributed through a sequence are a guaranteed sign that the sequence should be released from most research struggles. Overlong homopolymer sections (AAAAAAAAAA) mainly in the distal fragments of a sequence similarly hint at a substandard record (Nilsson, Abarenkov & Kõljalg 2016).

Caution should be taken when purely a partial number of fingerprints are combined in one analysis since clustering is likely to yield some sub specific

assemblies per species (Boekhout, Toen et al. 2002). Another reason to approve that, your fungi of expertise are orderly and correctly annotated in the sequence databases is that your research is expected to profit from it (Nilsson, Abarenkov & Kõljalg 2016).

Genetic Comparison Matrix; each of the samples is compared to all the other prototypes on the base of how many bands match. If all match then a score of 1 is completed. This is the case when a sample is compared to itself. Marks less than 1 direct that not all the bands match with a score of signifying, there is no corresponding bands between the two samples correlated (Linacre, A, Cole & Lee 2002). Lowest common ancestor method (LCA) and Tree- Bisection Reconnection (TBR) are two methods involved in clustering the candidate samples (Table 10.39).

Table 11.1 Sequences.docx Coverage of three regions for 96 samples
(means the average number of reads that align to or cover known reference bases)

Analysis stages details:

1) Trim primers; select the consensus sequences alignment, click on annotate & predict; trim ends

2) Import the raw data from MiSeq

3) Trim the raw data (from Miseq) with BBDUK trimmer

4) Map reference separately; select all trimmed data (aligned / assembled) (consensus options quality 250, call N if quality is less than 250) (discard trim annotations), consensus automatically created, select reference and then map to reference; call if coverage 5 options as our defined algorithm (for finding the reference go to Nucleotide (*Psilocybe*)

5) We extract ITS1 & ITS 2 manually form the samples showed just one ITS. We left the samples with 2 EF or no EF as it was

6) Reassembled with Denovo (separately); select mapped data to reference data; select 3 regions with diagonal line /// then assemble with Denovo. Select

the consensus sequences from reassembled with Denovo folder, then select workflow then select Batch alignment with MUSCLE (Align the consensus of each region with muscle alignment: select the consensus go to align/assemble:

multiple alignment: muscle alignment)

7) Download the RAXML & Mr Bayes from plugins in order to phylogenetic analysis, select the consensus alignment of each sample, go to tree, select the

RAXML, then ok

Select all samples from each folder of ITS1, ITS2, LSU and EF, group each region, then multiple alignment (together with any reference) (Best outgroup is reasonably closely related one to in-group)

8) Trim the actual binding site of primer manually

9) Concatenate the 4 regions together

10) From new alignment without N bases, make the Geneious tree (Geneious Tree

Builder)

(Diagrams 11.1., 11.2., 11.3., 11.4., 11.5., 11.6.)

A candidate species category for fungi should pass all these steps to classification. Out of 102 *Psilocybe* Fungi samples that were collected from Melbourne herbarium and genetically tested in this research, the current nominated species of 63 samples were confirmed, 31 unknown samples have been thoroughly identified through phylogeny method and received the name of their species (typed in red). Excitingly, one sample among the 31 had completely different species and made it uniquely special that it was the only species existing in Melbourne herbarium (highlighted yellow) (Table 11.3.).

Figures 11.1 (a-b-c-d-e-f-g-h-i-j-k-l-m-n-o-p-q-r-s-t) Geneious software analysis stages based on photos

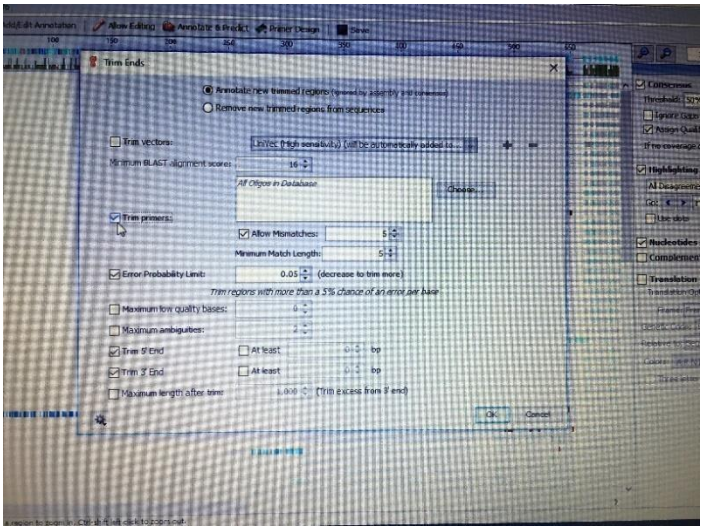


Figure 11.1a. Analysis with Geneious software

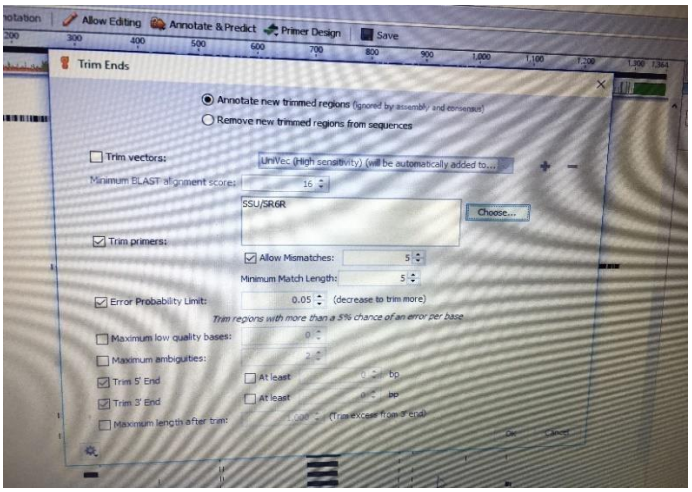


Figure 11.1b. Geneious analysis program

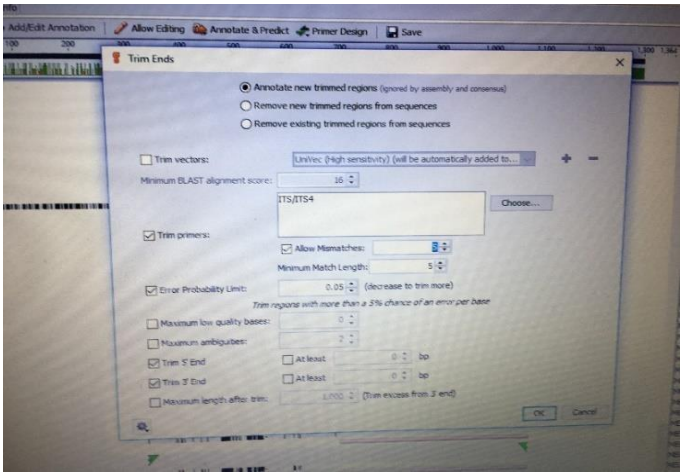


Figure 11.1c. Geneious analysis program

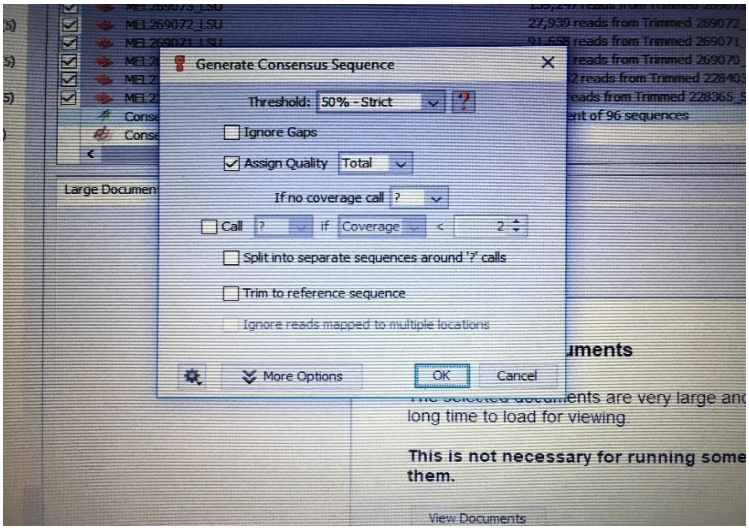


Figure 11.1d. Geneious analysis program

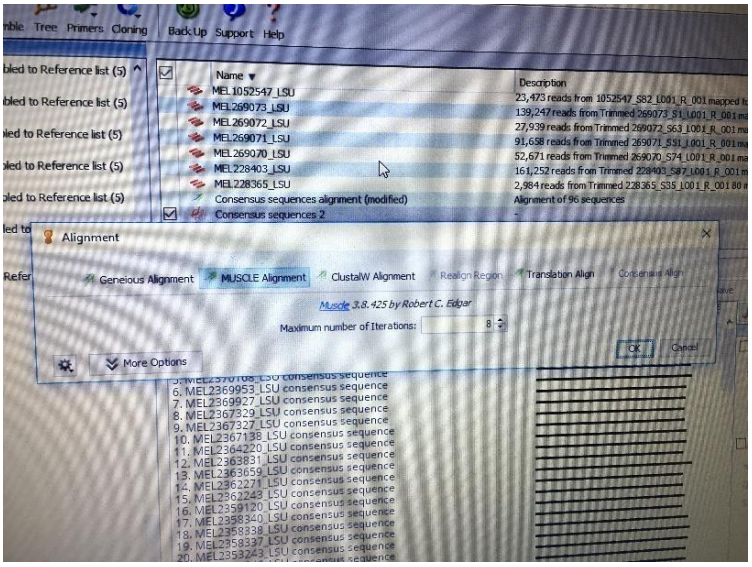


Figure 11.1e. Geneious analysis program

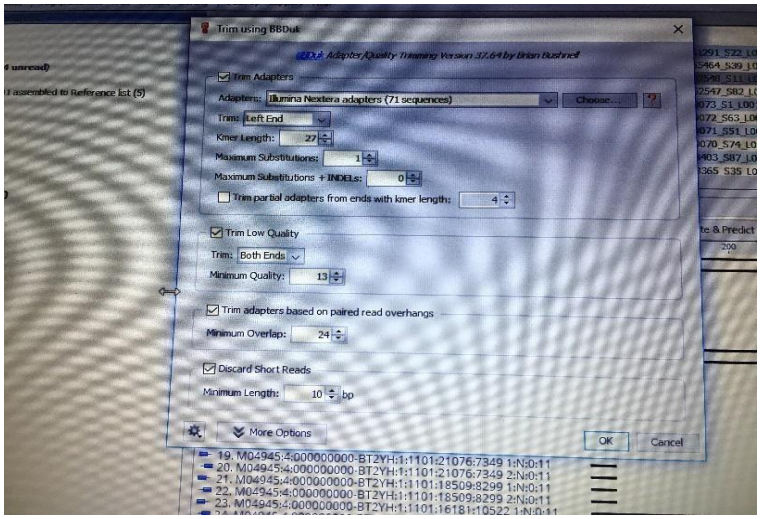


Figure11.1f. Geneious analysis program

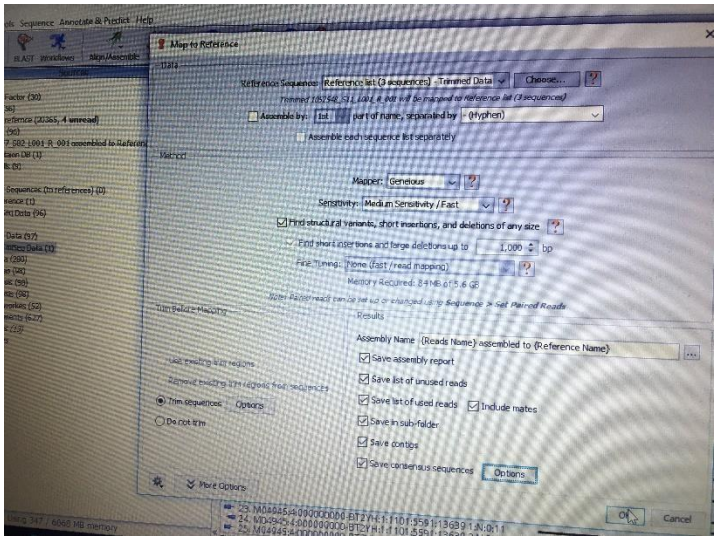
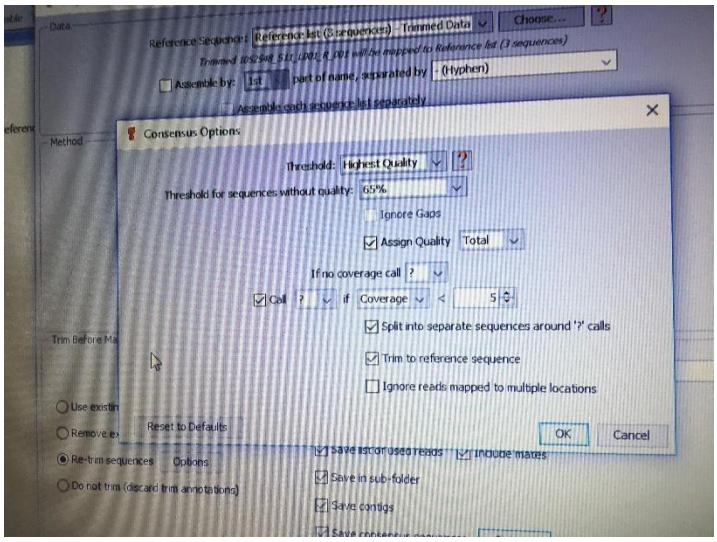


Figure 11.1g. Geneious analysis program



11.1h. Geneious analysis program

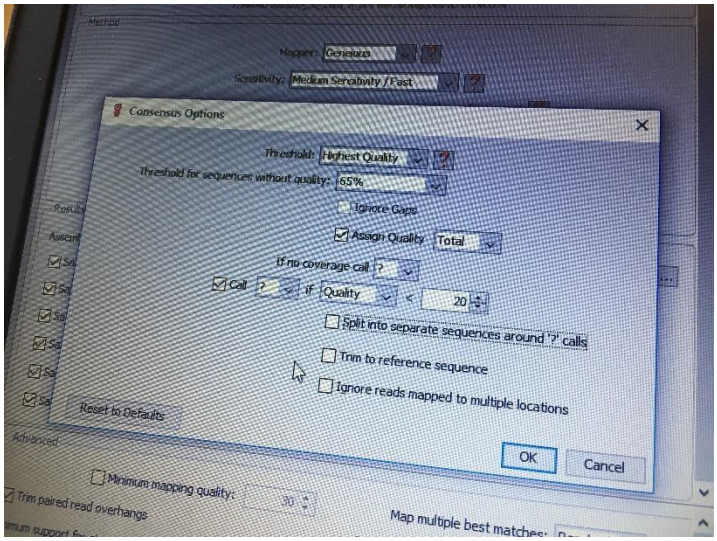


Figure 11.1i. Geneious analysis program

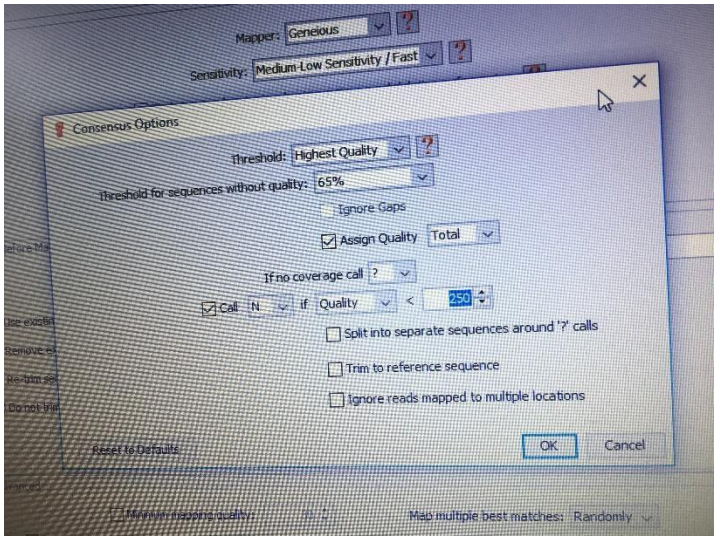
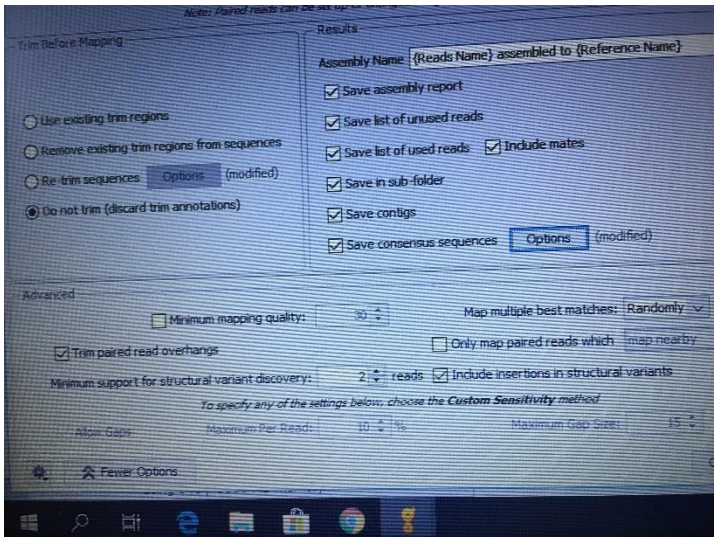


Figure 11.1j. Geneious analysis program



11.1k. Geneious analysis program

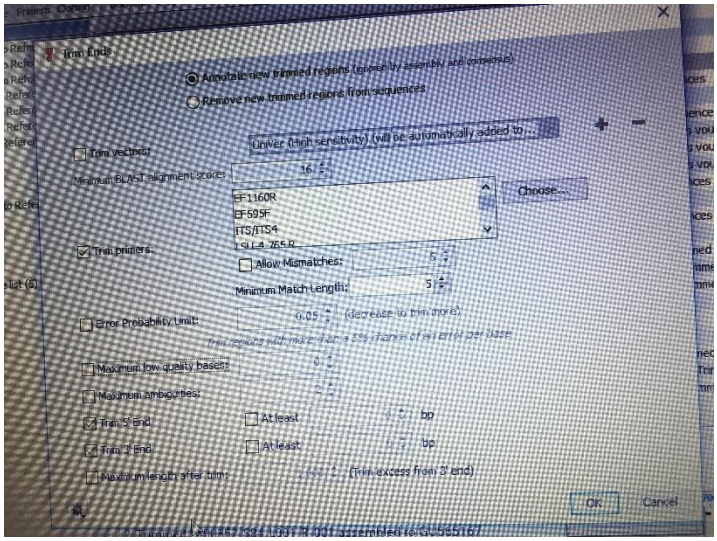


Figure 11.1l. Geneious analysis program

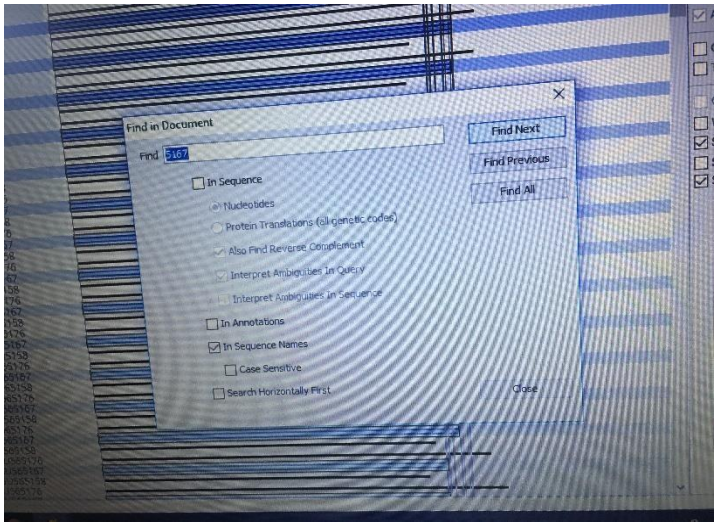
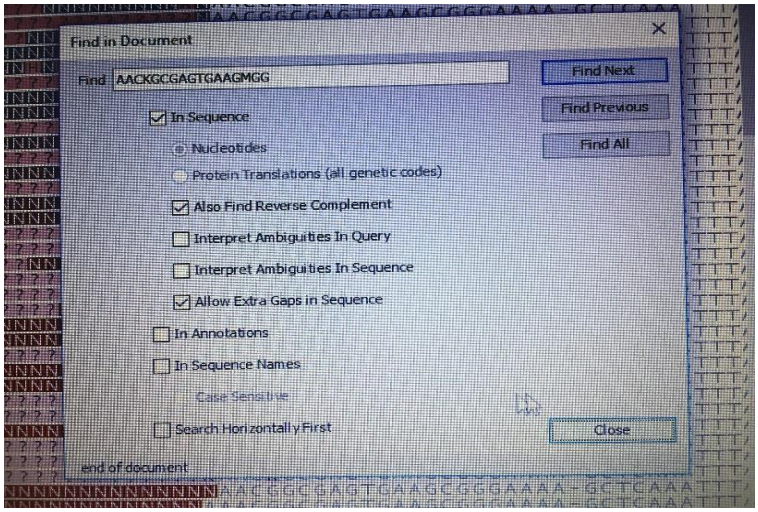


Figure 11.1m. Geneious analysis program



11.1n. Geneious analysis program

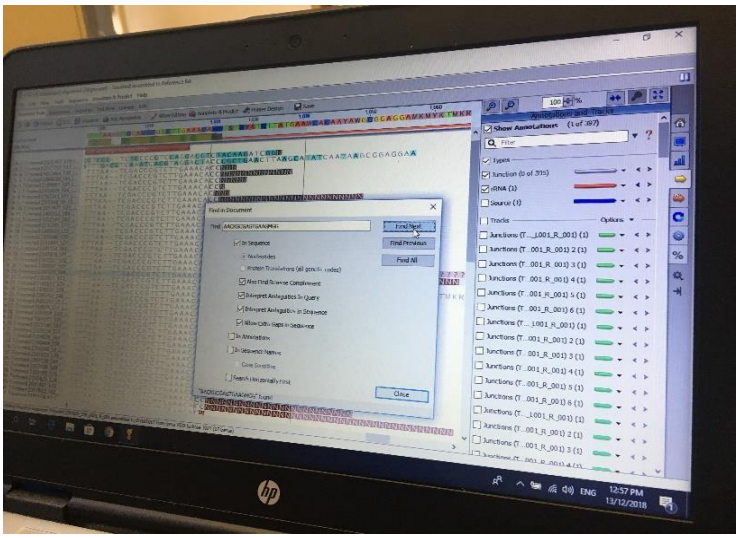


Figure 11.1o. Geneious analysis program

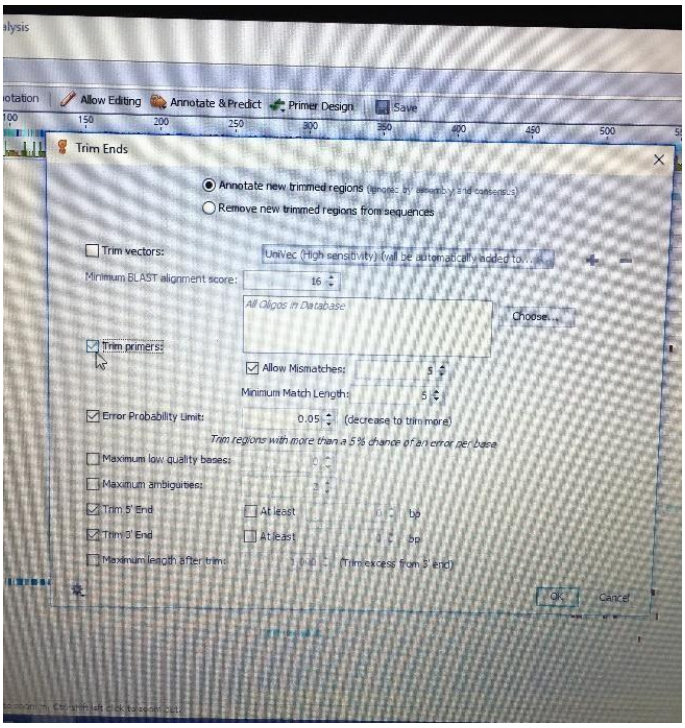


Figure 11.1p. Geneious analysis program

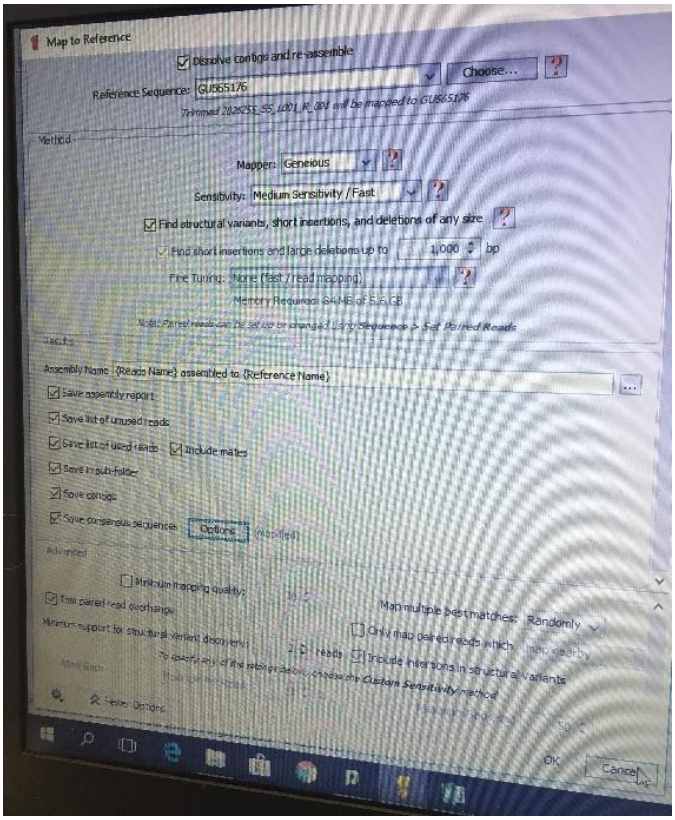


Figure 11.1q. Geneious analysis program

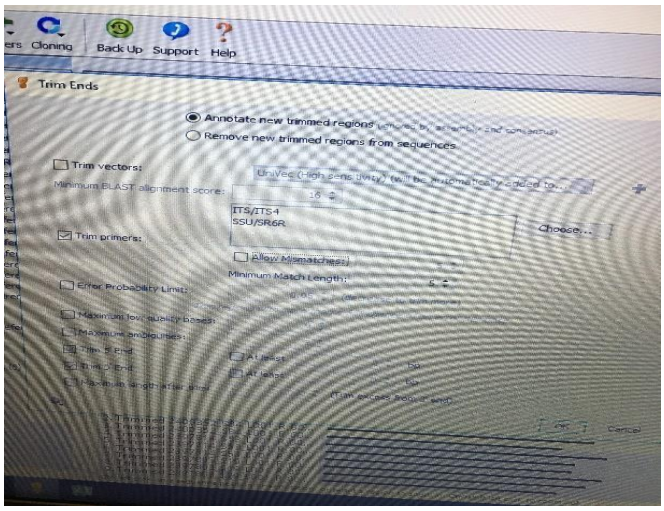


Figure 11.1r. Geneious analysis program

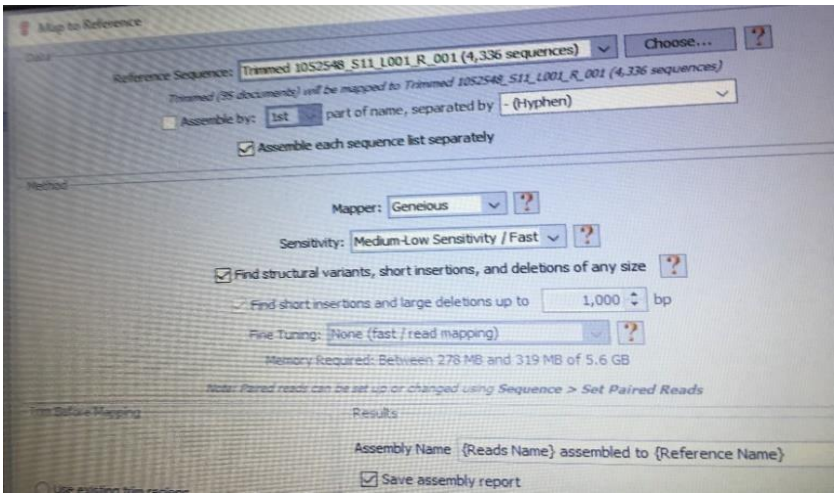


Figure 11.1s. Geneious analysis program

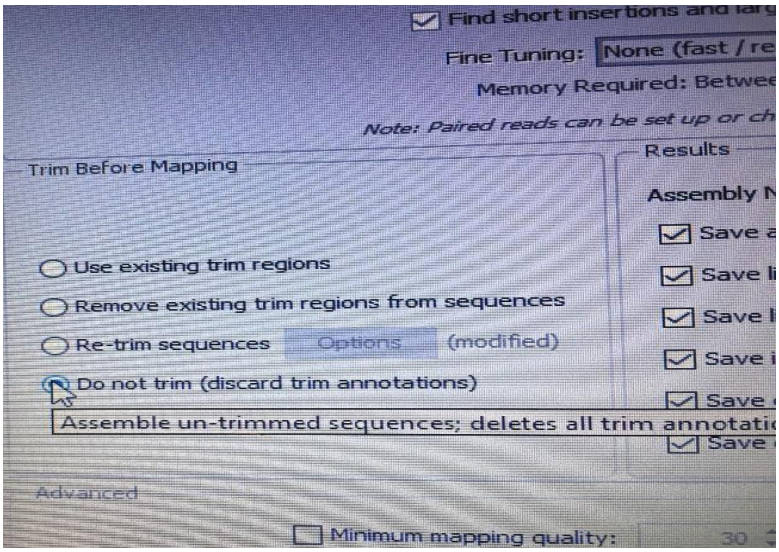


Figure 11.1t. Geneious analysis program

ITS1 (SSU)

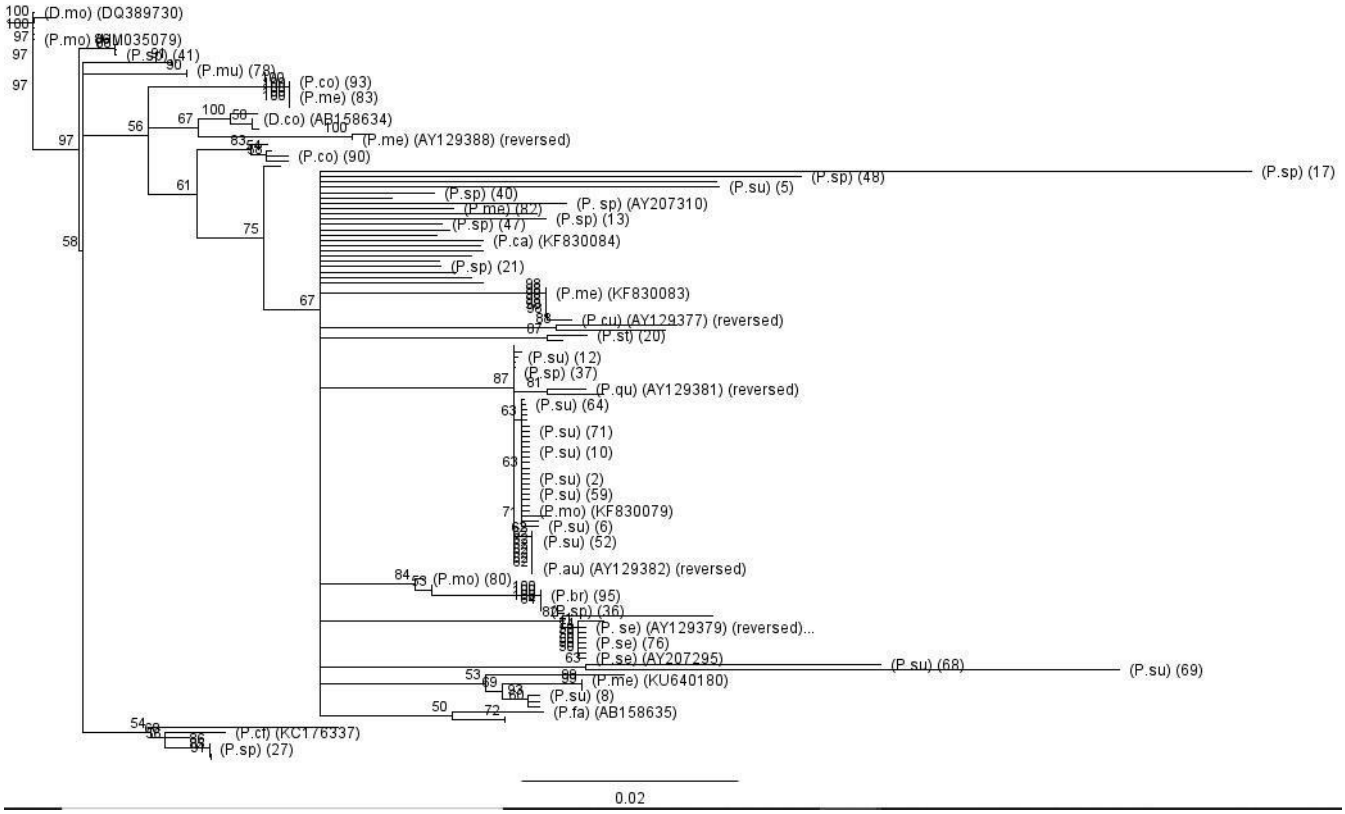


Diagram 11.1 Phylogeny tree of 96 samples for ITS1 region

ITS2

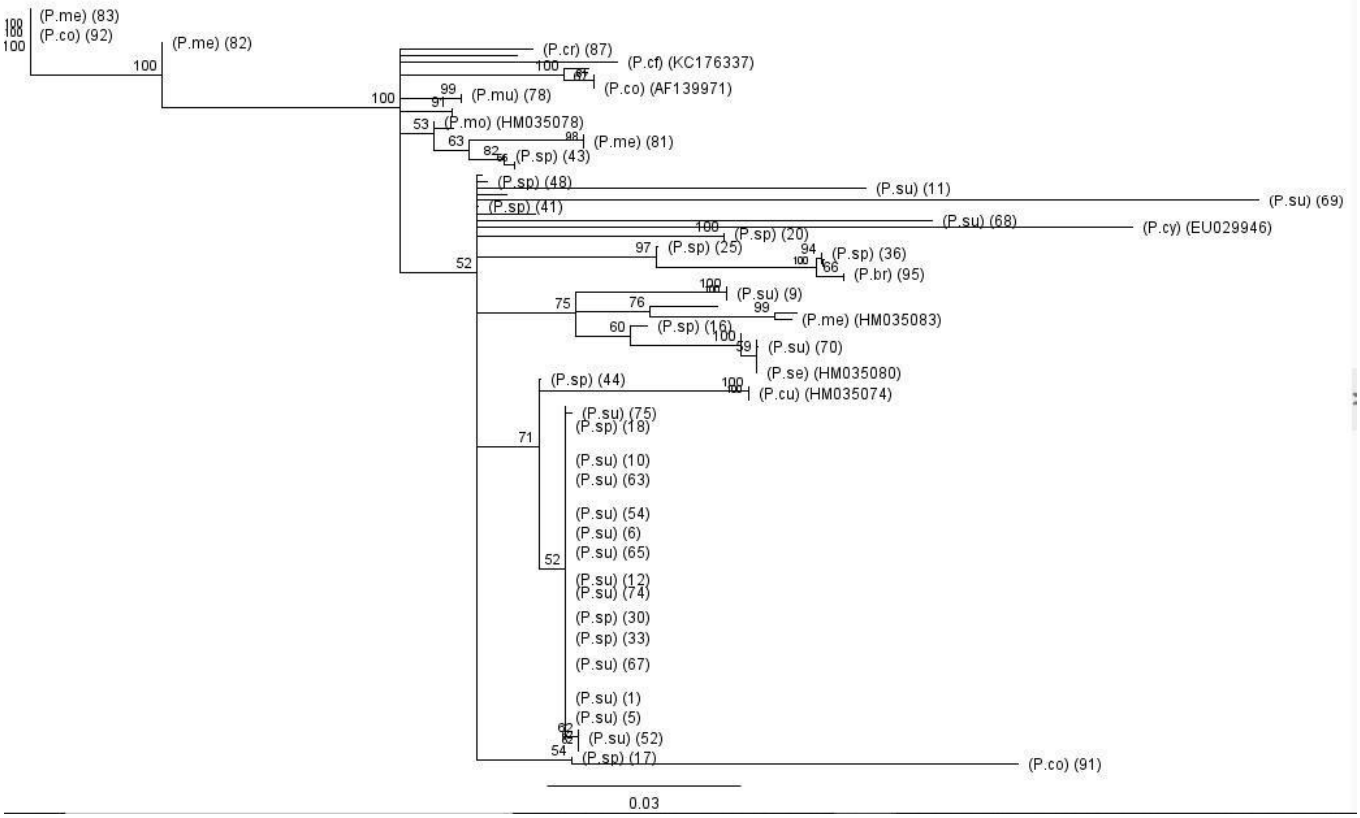


Diagram 11.2 Phylogeny tree of 96 samples for ITS2 region

(ITS3) LSU

Diagram 11.3 Phylogeny tree of 96 samples for ITS3 region

EF

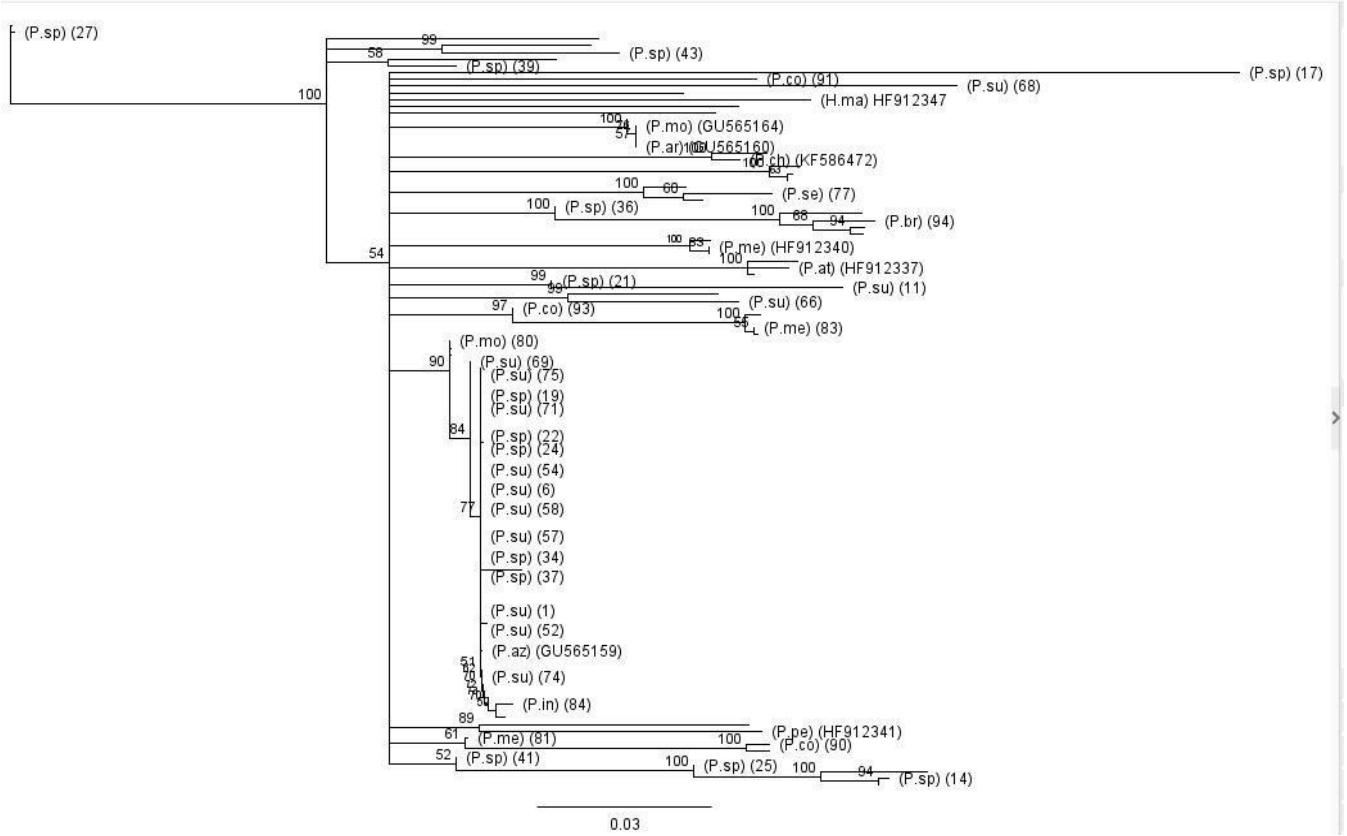


Diagram 11.4 Phylogeny tree of 96 samples for EF region

ITS1/ITS2/ITS3

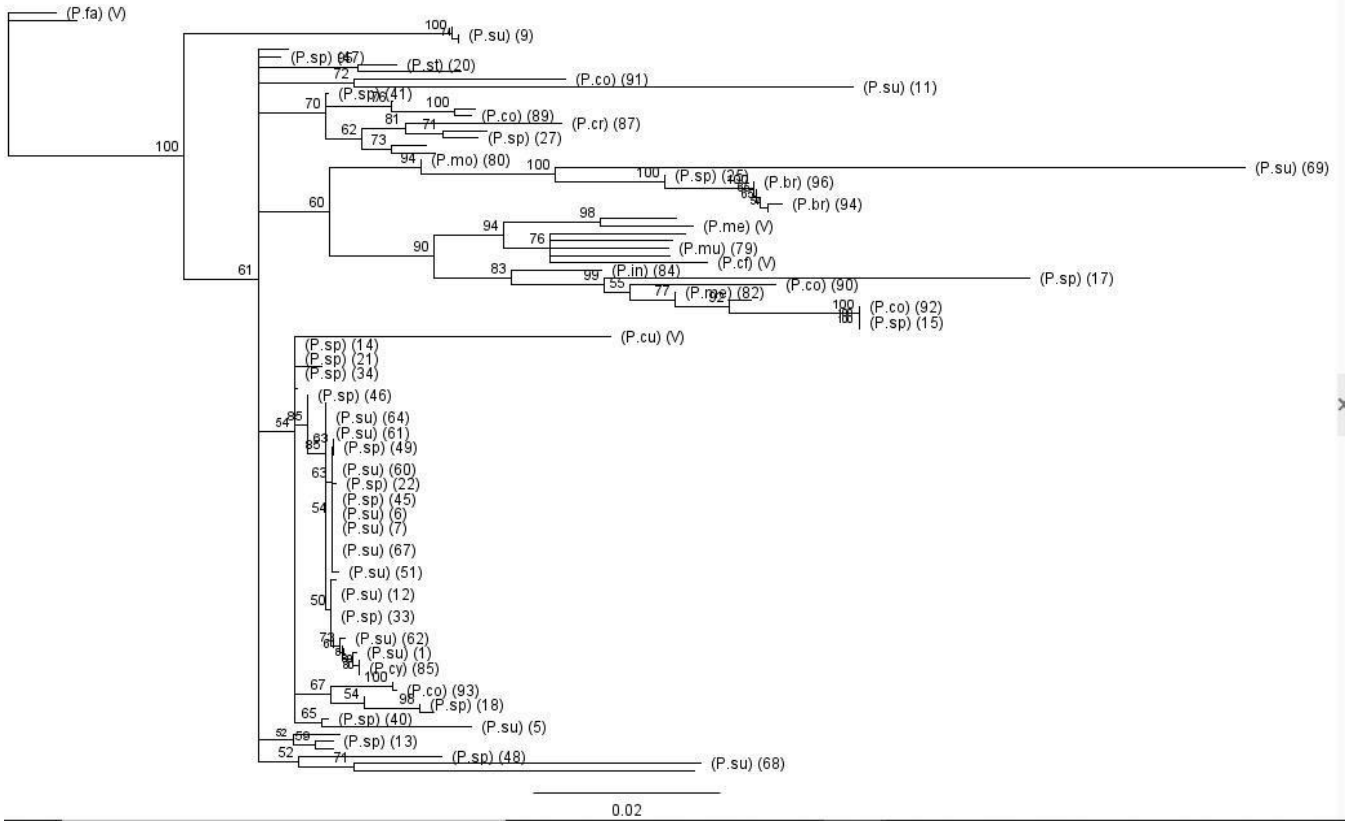


Diagram 11.5 Phylogeny tree of 96 samples for ITS1/ITS2/ITS3 regions

ITS1/ITS2/ITS3/EF

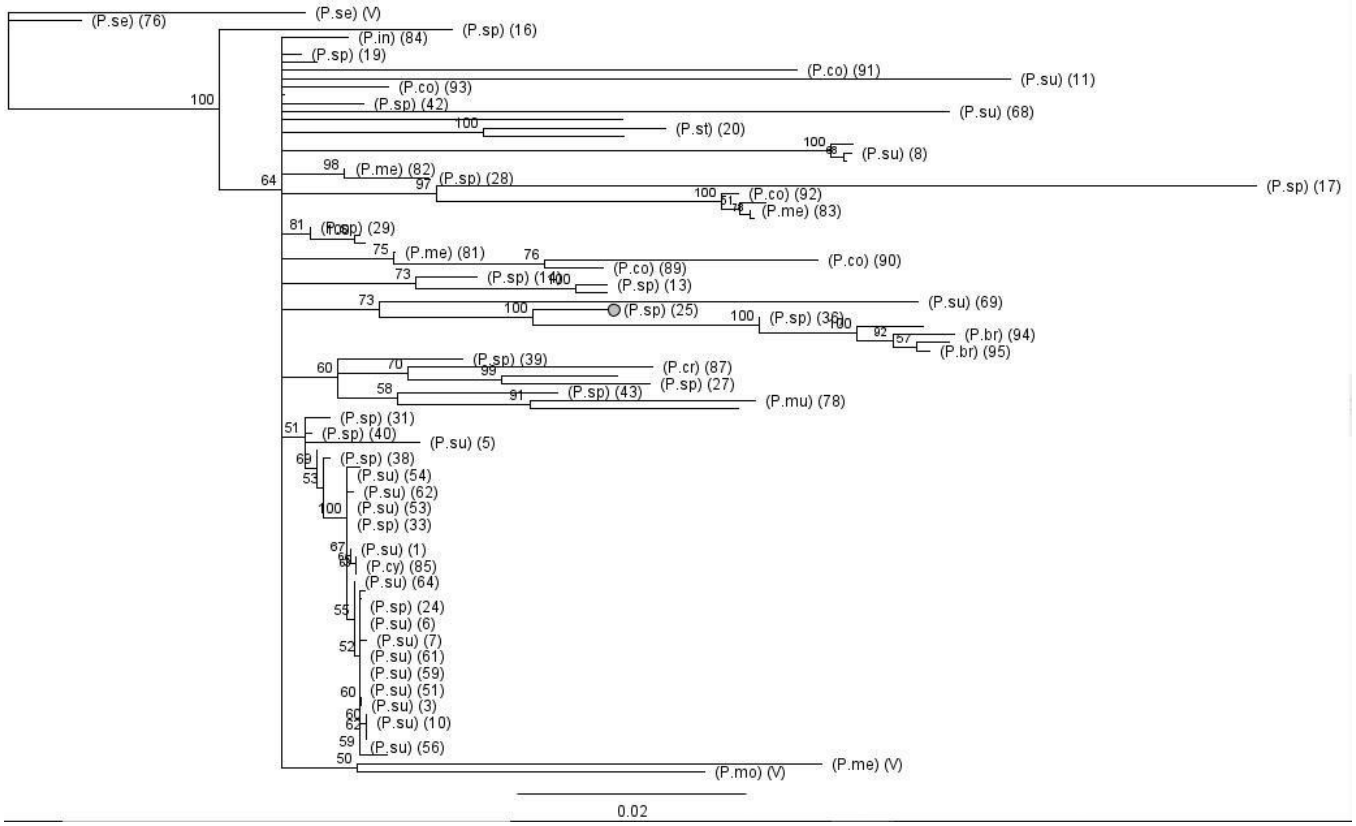


Diagram 11.6 Phylogeny tree of 96 samples for ITS1/ITS2/ITS3/EF regions

Table 11.2 Alignment of sequences.xlsx

Table 11.3 Results of the identified species (morphologically and molecularly) (Yellow highlighted row shows the unique species among the rest of samples in Melbourne herbarium)

	Catalogue no.	Genus	Species
1	0228365A	Psilocybe	Inquilina
2	2025140A	Psilocybe	Crobula
3	2025255A	Psilocybe	inquilina
4	1052548A	Psilocybe	Merdaria
5	2031497A	Psilocybe	stercicola
6	2031498A	Psilocybe	subaeruginosa
7	2031499A	Psilocybe	subaeruginosa
8	2031600A	Psilocybe	subaeruginosa
9	2032789A	Psilocybe	subaeruginosa
10	2039051A	Psilocybe	subaeruginosa
11	2053822A	Psilocybe	subaeruginosa
12	2054573A	Psilocybe	subaeruginosa
13	0228403A	Psilocybe	merdaria
14	0269070A	Psilocybe	merdaria
15	0269071A	Psilocybe	coprophila
16	0269072A	Psilocybe	cubensis
17	0269073A	Psilocybe	inquilina
18	1053363A	Psilocybe	?
19	1055464A	Psilocybe	coprophila
20	1052547A	Psilocybe	montana
21	2066942A	Psilocybe	subaeruginosa
22	2069119A	Psilocybe	subaeruginosa
23	2091051A	Psilocybe	merdaria

24	2095287A	Psilocybe	cyanescens
25	2103668A	Psilocybe	subaeruginosa
26	2103697A	Psilocybe	?
27	2103829A	Psilocybe	subaeruginosa
28	2104293A	Psilocybe	subaeruginosa
29	2104350A	Psilocybe	subaeruginosa
30	2112647A	Psilocybe	subaeruginosa
31	2121998A	Psilocybe	subaeruginosa
32	2151410A	Psilocybe	subaeruginosa
33	2231581A	Psilocybe	brunneoalbescens
34	2236445A	Psilocybe	subaeruginosa
35	2192209A	Psilocybe	subaeruginosa
36	2192210A	Psilocybe	subaeruginosa
37	2246934A	Psilocybe	subaeruginosa
38	2247018A	Psilocybe	crobula
39	2247029A	Psilocybe	Merdaria
40	2257903A	Psilocybe	subaeruginosa
41	2257957A	Psilocybe	subaeruginosa
42	2264832A	Psilocybe	subaeruginosa
43	2292425A	Psilocybe	coprophila
44	2297154A	Psilocybe	brunneoalbescens
45	2300488A	Psilocybe	musci
46	2300763A	Psilocybe	?
47	2305193A	Psilocybe	subaeruginosa
48	2305213A	Psilocybe	subaeruginosa
49	2305258A	Psilocybe	subaeruginosa
50	2305329A	Psilocybe	subaeruginosa
51	2305339A	Psilocybe	subaeruginosa

52	2314629A	Psilocybe	crobula
53	2316755A	Psilocybe	?
54	2316761A	Psilocybe	merdaria
55	2317863A	Psilocybe	subaeruginosa
56	2317392A	Psilocybe	subaeruginosa
57	2317408A	Psilocybe	subaeruginosa
58	2317471A	Psilocybe	merdaria
59	2317499A	Psilocybe	subaeruginosa
60	2317500A	Psilocybe	subaeruginosa
61	1061291A	Psilocybe	subaeruginosa
62	1061315A	Psilocybe	coprophila
63	1061294A	Psilocybe	subaeruginosa
64	2320533A	Psilocybe	coprophila
65	2320554A	Psilocybe	subaeruginosa
66	2320973A	Psilocybe	subaeruginosa
67	2321029A	Psilocybe	subaeruginosa
68	2321061A	Psilocybe	subaeruginosa
69	2321893A	Psilocybe	?
70	2321147A	Psilocybe	semilanceata
71	2322085A	Psilocybe	semilanceata
72	2192236A	Psilocybe	subaeruginosa
73	2335167A	Psilocybe	subaeruginosa
74	2335174A	Psilocybe	?
75	2335203A	Psilocybe	coprophila
76	2335646A	Psilocybe	brunneoalbescens
77	2336387A	Psilocybe	subaeruginosa
78	2341406A	Psilocybe	subaeruginosa
79	2341629A	Psilocybe	musci

80	2341799A	Psilocybe	subaeruginosa
81	2346803A	Psilocybe	?
82	2353240A	Psilocybe	cyanescens
83	2353243A	Psilocybe	semilanceata
84	2358337A	Psilocybe	subaeruginosa
85	2358338A	Psilocybe	subaeruginosa
86	2358340A	Psilocybe	crobula
87	2359120A	Psilocybe	subaeruginosa
88	2362243A	Psilocybe	subaeruginosa
89	2362271A	Psilocybe	subaeruginosa
90	2363659A	Psilocybe	caerulipes
91	2363831A	Psilocybe	coprophila
92	2364220A	Psilocybe	subaeruginosa
93	2367138A	Psilocybe	merdaria
94	2367327A	Psilocybe	subaeruginosa
95	2367329A	Psilocybe	musci
96	2369927A	Psilocybe	subaeruginosa
97	2369953A	Psilocybe	brunneo-albescens
98	2370168A	Psilocybe	subaeruginosa
99	2381037A	Psilocybe	coprophila
100	2397803A	Psilocybe	subaeruginosa
101	2397814A	Psilocybe	?
102	2400352A	Psilocybe	brunneoalbescens

12 Discussion (View of Concept)

In terms of evolutionary, Lamark's opinion is ladder form and Darwin's opinion is tree form. In 20th century the taxonomy needed to be reflected by evolution which means each living kind arose independently in its current form and is ongoing. Systematics is the study of the evolution of biological diversity and father of phylogenetic systematics is Willi Henning. The building of biological classification is Taxonomy. In fact, Taxonomy is the classification, identification and naming of organisms. Species delimitation is the act of identifying species level biological diversity. Practical species delineation approaches have broadly dedicated on four key area; discovering putative species, individual specimen assignment to a species group or operational taxonomic unit OUT, validation of candidate species or OUT as evolutionary distinctive lineages and inferring species relationship (Leavitt, Moreau & Thorsten Lumbsch 2015).

MOTUs that theoretically demonstrating new taxa, are regularly revealed in fungal molecular ecology studies. It is tough to track discovery of such unspecified entities (Hibbett, DS et al. 2011). MOTUs should be kept in close agreement with species (Ryberg 2015). Though acceptable taxonomic identification remains tricky in the kingdom fungi due to the vast mostly unexplored variety and the lack of reliable and abundantly annotated reference sequences (Kõljalg et al. 2013). There are no universally believed standard measures for MOTU delineation based on sequences. A cut off around 97% identity in the ITS region of the rRNA genes is repeatedly appealed for MOTU delimitation. The lack of a type specimen is a problem to official naming of MOTUs under the code (Hibbett, DS et al. 2011).

As genetic data have become easier and less expensive to gather, it has experienced a variety of methodological approaches to species delimitation. Transformed distance method and Neighbour relation method are methods for clustering. Distance methods determine the evolutionary distances between taxa and have less statistical power. Distance analysis estimates evolutionary distances which show the average number of substitutes per site in a DNA sequence. Pairwise distance differs the proportion of traits between taxa.

Adaptation is when a trait that evolved by natural selection for some biological function. Species have been modified by natural selection which is the main driver of adaptive evolution. Selection is tending to slow down the rate of evolution of positions that critically affect gene function. Gene is a piece of DNA that encodes a protein, gene sequence data is the main tool for reconstructing phylogenetic relationship and gene families are a set of related genes. Gene lineages can die but cannot be born. Sequences are the sum of all changes occurred during evolution and sequence alignment means correct position of gaps. Treelike history of a set of alleles or copies of a single gene is gene tree which shows gene genealogy. Diploid organism has two haploid genomes and two alleles. Phylogenies convert verbal or texture discussion of evolutionary relationships or classifications into lines. Homology is the evolutionary equivalent characters in different organisms. Formal mathematical models of morphological evolution have been developed. Principles of parsimony are minimizing number of gene duplication and gene deletion. Orthologue means no relevant gene duplication. Fossils are the organisms in present but stopped evolving long time ago. Ramification is the evolutionary common ancestry. Amount of morphological or ecological diversity within a taxon is Rank. Seed and pollen carry genes between distinct populations. Similar species may or may not share recent common ancestors but species that share a recent common ancestor tend to be similar. This information provides initial insights into the diversity of genes.

The members united by similar characteristics are polyphyletic group. A phylogeny is an explanation of how sequences evolved, their genealogical relationships and therefore how they came to be the way they are today. Phylogeny is actually the evolutionary trees and Consistency Index (CI) shows phylogenetic signal. Phylogenetic inference determines how long ago a pair of taxa last shared common ancestry and form Paraphyletic groups. A phylogenetic tree shows the inferred evolutionary relationships among various biological species based on similarities and differences in their physical or genetic characteristics. These relationships are discovered through phylogenetic inference methods such as DNA sequences. Poisson Tree Process (PTP) model to infer putative species boundaries on a given phylogenetic input tree. All the descendant of a single ancestor consists monophyletic group (Clades are the

monophyletic groups). Monophyletic taxa is the taxa that accurately reflect evolutionary relationship. In the trees system the highest means the earliest in terms of time and shorter trees, more likely to be true than longer trees. The more parsimonious is in the shorter distance and the shortest tree is the most parsimonious tree (PAUP) and the best one too. The least informative tree is the longest one. Each position in the sequence is a character and character state changes at the nodes on the tree. Alternative versions of the character form character states and different character states form Polymorphic. By moving away from the optimal tree, the likelihood of probability becomes lower. Phylogenetic trees framework is based on studying the evolution of traits. In tree with n taxa, total probability of the trait data is 2^n. Conflict among the gene trees is expected to be due to recombination among individuals within species. A set of organisms living at a certain time with potential interbreed, constitute sexual population. Homologous gene copies on different chromosomes as well as sequence variants segregating in a population are, alleles. Diploid sexual organisms have two alleles; one mother and one father, maternal allele is egg and paternal allele is sperm. Homozygote means that two copies of alleles / characters conflict and lead to overlapping taxa. When all alleles at a locus are homologous, it is single locus. Changes by chance in allele frequency is genetic drift. Fixed derived allele is irreversible. Parent-offspring connections are like glue that hold population. During DNA replication, nucleotide positions in offspring from parents are Homologous. Chromosomes assort independently during meiosis phase. Mutation (inversions, translocation, indels: insertion / deletion) is when common base replaces by itself and no actual substitution. μ is the mutation rate per minute. Different characters have dissimilar combination of alleles and diverse genes have different genealogical within a species (Taylor et al. 2000).

Expression of organisms' genetic makeup, forms Traits (characters / characteristics / phenotypes). Morphology is in fact, physical make up. Every living organism has one or two direct ancestors although taxon members have common ancestor and many traits in shared. Common ancestry is the opposite of separate ancestry. Inference is the informed conclusions guided by observational evidence. Pairwise alignment just involves two sequences but multiple alignment

can cover many taxa. Homology is an inherently phylogenetic concept which considers relationship between traits in different taxa (Taxon's plural is taxa) and based on common ancestry not similarity, but Homoplasy is the relationship between traits variation. Traits change over time and Homologous traits trace to the same trait in a common ancestor. As times continues more, changes will fail to generate additional differences. In slowly evolving regions (conserved domains / coding regions); traits evolve more slowly and provide more reliable phylogenetic evidence. Derived character states are called Apomorphies which is novel characters while Synapomorphies are shared derived traits. Homoplastic characters are similar characters that are not derived from a mutual ancestor (Leavitt, Moreau & Thorsten Lumbsch 2015).

Accumulated changes along the lineage are leading to living species and lineage splitting (groups isolated from one another), creates speciation event. Speciation takes place as a result of Allopatric (allo=different, patria=homeland) separation. Lineage fusion is the complete merging of two distinct population and closer means separated by fewer lineage splitting events. Biological species concept is the interbreeding with one another in species. DNA sequence is the character states and nucleotide position (AGCT). Transitions tend to happen at a higher rate more frequently than transversions. States of DNA: AGCT (Purines (A / G) and Pyrimidines (C / T)) (Transition: purine to purine or pyrimidine to pyrimidine. Transversions: purine to pyrimidine or pyrimidine to purine). Consistence is only one change in character state and Consistency Index (CI) is the minimum number of changes. Members of the same species are conspecific. Additionally, there is general agreement that species should not be recognized solely on the basis of sequence similarity in so far, as supplementary related characters are obtainable or could be regained (Hibbett, DS et al. 2011).

Although studies on a range of non-mental health issues including some cancers, where treatment using fungal-based compounds have been carried out, these studies are less familiar (penicillin excepted) but have had notable clinical and real-life impacts (Grob, Bossis & Griffiths 2013). There are different medicinal applications for members of the genus *Psilocybe* around the world, based on their chemical components (Moreno et al. 2006). Psychoactive agents like psilocin, psilocybin, ibotenic acid and moscimol pass through the blood – brain

barrier and act on neurotransmitter receptors (Matsushima et al. 2009). Even though the possible use of the compounds, found in the genus *Psilocybe* have the potential for more widespread use and application, the development of these useful compounds is limited by the reliance on wild-source materials that lack quality control and full characterisation of their chemical profiles and concentrations. Initial work first started in the 1950's showed clear clinical applications of the chemical compounds found in *Psilocybe,* but little work has been done to determine and clarify potentially useful or useless taxa and potentially dangerous ones (Grob, Bossis & Griffiths 2013) particularly in Australia.

The results from maximum parsimony analysis are corroborated by the similar topology obtained, using the maximum likelihood analysis. Sequences obtained from Denaturing Gradient Gel Electrophoresis (DGGE) bands are rather challenging to analyse, as they are generally from different orders and classes. Our taxonomic knowledge is still poor and phylogenetically, most of the sequence types do not fit obviously within any known family / genera or species, though their ordinal classification seems to be reliable. Definitive species identification is very difficult, even a large number of representatives are accessible from databases and adequately variable gene regions are analysed. Most of the available sequences and phylogenies are derived from the rDNA gene, but classification and taxonomic patterns based on this gene alone are inadequate, subject to debate and need to be re-evaluated.

Definite and clear identification of the taxa in the fungi genus *Psilocybe* based on two methods of identification will be essential to allow full development of standards and implementation of testing methods by national and international drug control legislation. Identification of particularly useful species will allow for the development of lab-based populations of known high quality 'clones' that can be further developed for clinical trial and use. Depending on the outcome of any drug trial, the identification of morphologically and genetically of species of *Psilocybe* from Australia could lead to new patentable drugs. The discovery, development and use of products sourced originally from wild fungi are the ultimate goal of any Ethnomycologist!

Apart from the general human use of consumption for food, fungi of various types have been widely but tentatively applied in medicinal settings. Most notable studies have investigated the use of fungi-derived chemical compounds in psychoanalysis and treatment to assist in the breaking of a person's habitual experience of the world. These compounds can help patients caught in an ego centred problem cycle and assist them in release from their fixation and isolation (Schultes, Hofmann & Rätsch 2001).

This research is classified as Ethno-directed research. In this study, we performed molecular identification of the species complex based on sequences of the internal transcribed spacer (ITS), translation elongation factor 1-α (TEF1), and SSU/LSU regions. Late 1970's, first generation of DNA sequencing has been occurred. 2000's is the era for second generation of sequencing. The expertise belongs to the third generation of DNA-sequencers promise DNA-reads of 20 Kbp or more (Faure & Joly 2015).

The use of molecular data has led to an improved perspective on the taxonomic value of many phenotypic characters in lichenized fungi and species delimitation in general. Fungi are a perfect model for evaluating variance in eukaryotes due to their simple morphologies, small genomes, broad ecological roles and diverse lifestyle. Traditional phenotype-based approaches to species recognition act to massively underestimate diversity in lichen-forming fungi. Whereas molecular research has supported traditional phenotype-based hypothesis of species boundaries in a number of case studies, repetitively demonstrate that our existing explanation of morphological and chemical characters often fails to perfectly characterise species-level diversity (Leavitt, Moreau & Thorsten Lumbsch 2015).

In some point that it is not easy some times to distinguish the margins of these two majors. It is not anticipated that lately inherently isolated species direct phenotypic variances, though over time they should (Taylor et al. 2000). Most species delimitation methods based on single-locus sequence data fall under two general categories either genetic distance or tree-based tactics. Tree based species delimitation methods can likewise be used on the basis of other properties related to phylogenetic tree topologies. A number of tree-based

methods, partly automate the species delimitation process with definite bioinformatics analysis (Leavitt, Moreau & Thorsten Lumbsch 2015).

This study can be classified in Human Microbiome Project (HMP) (research on the microbiome and its role in human health and disease) and is very valuable in human mental problems. Several recent studies have presented that molecular identification can be positively used for fungi (Inyod et al. 2017). Other studies show that the high grade of polymorphism of sequences from the ITS region may lead to unclear identifications of thoroughly linked species (Kowalczyk, M et al. 2015). Recent studies based on environmental sequences have detected several formerly unknown fungal lineages, so representing that fungal diversity is perhaps much higher than currently known (Truong, C et al. 2017).

Molecular techniques have shown to be more reliable in the identities of wild collection and are helpful in mushroom taxonomy**.** The status of complexity and misapprehension makes us to approach the identification with morphological and molecular skills (Cheng et al. 2008). The situation is further complicated by the fact that very few specific type collections had been sequenced, so that standard references and universally valid, are lacking. This research considers the prospects for sequence-based taxon discovery and explanation over analysis of environmental sequences (Hibbett, DS et al. 2011).

Great development has been made with the use of modern molecular methods in the taxonomy and phylogeny. Molecular identification in mushrooms has become progressively important, since it can overcome the limitation of morphological identifications in numerous cases. Accumulated DNA sequence information, in particular DNA barcoding using the ITS region, has greatly improved accuracy in identification of fungi at the species level and improved our understanding of fungal phylogenetic relationships. The use of 16S rRNA gene sequence data is reformed our understanding of the microbial world and led to a fast surge in the number of descriptions of novel taxa, specifically at the species level. This verdict supports the fact that both ITS and nLSU can perform similar work as DNA barcode, but ITS data set are commonly superior to LSU in species discrimination (Hussein et al. 2014). ITS often powerfully supports phylogenetic

species, but fails to provide robust resolution of the branching order among those species (Frøslev, Matheny & Hibbett 2005).

We recommend three wide-ranging parts of research to increase the helpfulness of fungal DNA barcoding to meet the current and coming challenges; 1) develop a common set of primers and technologies that allow the amplification and sequencing of all fungi at both the prime and secondary barcode loci, 2) collect a centralized reference database that embraces all accepted fungal species as well as species hypothesis and permits consistent updates from the research community and 3) form a consensus set of new species recognition conditions based on barcode DNA sequences that can be applied across the fungal kingdom. In addition, DNA sequence comparisons among existing species have demonstrated that many traditionally used phenotypic characters in fungi do not essentially predict phylogenetic likeness. However, it should be noted that fungal species identification based on DNA sequence information at one or two barcode loci diverge from the current gold standard for fungal species recognition that operates the phylogenetic approach and is based on the concordance of multiple gene genealogies (James 2015).

Over the last 50 years, DNA–DNA hybridization (DDH), which measures indirectly the degree of genetic similarity between two genomes, has been the ‘gold standard’ for bacterial species segregation by providing a persistent numerical threshold (DDH value 70 %) for the species boundary. Before the dawning of the molecular biology era, we were very slow in discovering novel bacterial and archaeal species due to the absence of effective and objective methods to spot and delineate novel species. In 1992, Fox and colleagues first observed two bacterial strains fitting to different species which can share high levels of genetic similarity. Even almost equal 16S rRNA gene sequences, if two strains share less than 97% 16S rRNA gene sequence similarity, they fit to different species. In genome-sequencing projects based on the Sanger method, depths of sequencing coverage generally range from 7- to 10 - fold (one nucleotide position of the genome is sequenced seven to ten times on average). In compare, at least 306 depths are typically applied in NGS based genome sequencing projects. The only cost-effective way to find such a rare species or stages in mass sequencing is via NGS barcodes (Meier et al. 2016). Each of the

accessible NGS technologies has pros and cons regarding read length, accuracy, the nature of sequencing faults, its capability to produce paired-end information and cost-effectiveness. Next gene sequencing is used to evaluate genetic diversity. Secondary signals owing to heteroplasmy and / or pseudogenes hardly lead to sequencing failure in NGS. NGS barcoding needs simply the subsampling of a specimen through the removal of a small amount of tissue. NGS is more sensitive to contamination than Sanger sequencing and cross-contamination is more common even between specimens from the same samples. NGS barcodes offer assistance by lowering the charge of barcodes by > 95% and the procedure is cheaper than organizing by Para-taxonomist. NGS barcodes therefore link the growing list of tools that can aid taxonomy. NGS has been recognized as a potential tool for the analysis of bulk samples in molecular ecology (Meier et al. 2016).

NGS allows an enormous production of DNA sequences (Faure & Joly 2015). Specimens with low amplicon yield, are simply recoverable due to the increased sensitivity of the NGS approach (Shokralla et al. 2014). One of the most broadly used applications of NGS is Metabarcoding which uses a nominated DNA sequence reads into (OTUs) and helps to classify the closest known species. Despite its technical practicality, this approach to diversity exploration is still restricted by some issues including the method of DNA isolation, selection of marker / PCR primers and analysis of the data (Větrovský et al. 2016). Minimum standards for naming MOTUs might contain the following; 1) demonstration by at least two full length sequences of a proper genetic marker perfectly consistent with the future barcode standard for fungi and derived from liberated studies with one sequence, 2) a published phylogenetic analysis representing monophyly and considering all related openly available sequences repossessed using Blast or a similar method, 3) application of chimera testing software and other quality control measures, 4) locality data at least for one sequence and 5) registering a taxonomic database (Hibbett, DS et al. 2011). DNA barcoding is an effective technique to classify specimens and to spot undescribed / cryptic species. Sanger sequencing of single specimens is the normal method in creating DNA barcode libraries and identifying unknowns.

However, the Sanger sequencing technology is in some respects inferior to next generation sequencers which are accomplished for generating millions of sequence reads concurrently. Moreover, direct Sanger sequencing of DNA barcode amplicons as practised in most DNA barcoding procedure, is troubled by the need for comparatively high-target amplicon yield. Co-amplification of nuclear mitochondrial pseudogenes confuse with sequences from intracellular endosymbiotic bacteria and examples of intraindividual variability. Apart from low output, Sanger sequencing needs a DNA amplicon template of high concentration (100 - 500ng) to avoid characteristic biases and mistakes. Sanger sequencing affords a single sequencing signal form or electropherogram for each sequence created. The presence of DNA sequence signal from a low concentration challenging source can often cause sanger-based sequencing of an organism to fail to generate the real DNA barcode (Shokralla et al. 2014).

The General Lineage Concept (GLC) highpoints that no solo property should be considered as describing for the recognition of species apart from developing lineages and segments of meta-population ancestries. The GLC allows investigators to delineate unclear species boundaries, using different properties and simplifies the development of new methods to check hypothesises of lineage separation (Leavitt, Moreau & Thorsten Lumbsch 2015). In our view, this indicates that at least those noticeably genetically isolated lineages that can be distinguished by rather than molecular characters should be treated as different species (Lutz et al. 2005). By means of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) we can only define in the present and we cannot predict the future (Taylor et al. 2000). GCPSR is normally applied in mycology. The review reveals that PCR amplifications or ribosomal RNA genes are more reliable across the fungi than the protein coding markers (Schoch et al. 2012).

The use of molecular identification is fast, sufficient, reproducible, and can provide high specificity to distinguish between the species and subspecies (Species; a solitary lineage of ancestor descendent populations which preserves its uniqueness from other such lineages and which has its own evolutionary tendencies and historic fate) of fungi unlike the morphological and biochemical tests, used in the laboratory diagnosis. Species concept is for the theoretical variety and Species recognition is for the operational ones. Unluckily when it

comes to classifying species, the Evolutionary Species Concept (ESC) is not useful since it has no recognition standards. In compare, the many secondary operational species concepts do identify conditions for diagnosing species. Under the ESC, species have been recognized by morphology biology and phylogeny but our understanding of fungi is that the phylogenetic analysis of variable nucleic acid characters presently comes closer than the others to spotting species consistent with the evolutionary species concept. Phylogeny achieves best, because one progeny evolutionary species have shaped from an ancestor, changes in gene sequences occur and can be documented before changes have occurred in breeding behaviour or morphology (Taylor et al. 2000).

In fungi, biology (Biology; sets of truly or possibly reproducing natural populations which are reproductively isolated from other such group) has been used to classify groups of mating compatible individuals which have been paralleled with species. However mating tests are impossible to apply to fungi that lack meiospores. This problem is a thoughtful one because roughly 20% of fungi are morphologically asexual and do not produce meiospores. What are measured to be biological species, using biology would be reflected to be different phylogenetic species using phylogeny, if the gene is polymorphic within a species or fixed for alternative alleles in two species (Taylor et al. 2000).

The species unit is essential in biology. Species are the primer measure of diversity and the unit of many philosophies and subjects (Ryberg 2015). The fungal high throughput taxonomic identification implements for use with NGS (FHiTHINGS), a new open-source data treating tool that quickly simplifies the identification and taxonomic classification of fungal ITS sequences database. It has capability to multiplex a great number of samples within a solo sequencing run database. FHiTHINGS offers some chief developments in the taxonomic classification of fungal ITS sequences (Dannemiller et al. 2014).

Distances among organisms belonging to the same species are smaller than distances among organisms from different species. Genetic distance approaches hold certain promise as an identification tool, shortcutting the complications of morphology-based identification, though in practice, a barcode gap may not exist for many groups. Moreover, the role of distance-based approaches using a single

genetic marker for species discovery remains more debatable (Leavitt, Moreau & Thorsten Lumbsch 2015).

We employed benchtop sequencing platforms (MiSeq) to recover 600 bp DNA barcodes of 96 specimens, using 25% capacity of a MiSeq run (i.e., two lanes of a 16-lane run). In a subset of samples, we also detected non target species and heteroplasmic sequences. Around 80% of respondents, stated no problems with PCR amplification of ITS, 90% scored it easy to achieve a high-quality PCR product and 80% reported no significant sequencing concerns. In comparison >70% reported PCR amplification problems for RPB in which primer failure as the major problem (Schoch et al. 2012). Illumina NextSeq sequencer is announced, combining a high capacity and comparatively low costs (Zoll et al. 2016).

The species delimitation plug-in of the Geneious software runs statistical approaches for determining species boundaries in single-gene topologies. An important input of the species delimitation plug-in is that, it delivers an objective means for operators to evaluate putative species within a practical statistics-based outline rather than qualitative evaluation of what level of hierarchy constitute a species in phylogeny. Eventually Geneiuos is a firm program that can be used to group individuals signified in a single-locus sequence alignment into candidate species that should be supplemented with other lines of evidence in an integrative taxonomic style. Average nucleotide identity (ANI) signifies a mean of identity values between multiple sets of orthologous sections shared by two genomes. Another restriction of clustering approaches is that, they do not assess or take into account evolutionary divergence and interactions among population clusters. Hence a probable functioning protocol for an informed species delimitation study that takes into account population structure, could consist of first applying a genetic clustering analysis under a population genetic criterion to classify genetically distinct population clusters that can be measured candidate species. Generalized Mixed Yule Coalescent GMYC is a quantitative method that measures the degree of mtDNA genetic clustering. From these candidate species, a species tree can be inferred for focal group, using coalescent-based species tree reconstruction methods. Consequently, a coalescent-based validation method can be applied to assess, whether the distinct population clusters represent independent evolutionary lineages (Leavitt, Moreau &

Thorsten Lumbsch 2015). Using GenBank for analysing the data through Geneious program shows that the global data which is coming from GenBank can be the benchmark for our findings as we derived our results based on the whole existing data in the GenBank and against whatever is registered in GenBank.

Bootstrap (BT) is the most widely method evaluating strength of clade support and the way to assess how likely our conclusions are sound. Bootstrap scores (bootstrap percentage) are 0-100%. Parametric bootstrapping is much laborious than nonparametric and tests specific phylogenetic hypotheses, for example maximum likelihood analysis but non parametric bootstrapping is like many different methods of phylogenetic analysis. Chimeras are synthetic sequences composed of two or more sequence fragments that do not fit together but that are merged in the PCR or sequence assembly stages. The archetypal chimera is formed when the PCR enzyme shifts template from one species to the other in diverse template PCR reactions. Any company of a preserved sequence piece in the objective marker such as the 5.8 S gene in ITS sequences rise the risk of chimeric unions. Chimeras lack a significant genetic clarification and lead noise and bias to studies featuring them. Molecular identification, richness approximations compound, sequence alignment and phylogenetic inference are examples of processes that are adversely affected by chimeric sequences. Steps can be taken to ease the chances of Chimeras formations in the PCR stage. Similarly, vital is to monitor for chimeras in freshly generated datasets. This should be done on a routine base for all new fungal ITS datasets (Nilsson, Abarenkov & Kõljalg 2016).

Genetic segregation leads the loss of common polymorphisms (Taylor et al. 2000). Some species-level lineages are likely covered of chemically and morphologically polymorphic individuals which are conservatively considered as distinct species. Distinctive species do not need to have diagnosable morphological differences. However, in practice, a pure separation between intraspecific and interspecific variant is commonly subject to observation bias and individual clarification (Leavitt, Moreau & Thorsten Lumbsch 2015). DNA based species identification depends on distinguishing intra-specific from interspecific variant. The varieties of these sorts of variation are unknown and might differ

between taxa (Purty & Chatterjee 2016). Most of the studies on the Ascomycota and the Basidiomycota have stated a within-genome changeability lower than 3% which corresponds to the typical intraspecies deviation in this group (Větrovský et al. 2016). Chronograms which contain information on time can be very useful for tracking changeability.

My work fits into the previous studies using crossing tests to delimit species show that, there often is an overlap in within and between species distances. Some scientists do not view species as central units but randomly delimit taxa in a phylogenetic hierarchy (Ryberg 2015). None of the techniques of species recognition can recognize the instant that, individuals in an ancestral species are splitting into offspring species (Taylor et al. 2000). With assessing species boundaries, putative species are delimited using both morphology and molecular data. Percentage of consigned reads, declined going down alongside taxonomic levels from phylum to genus (Kingdom / Phylum / Class / Order / Family / Tribe) (Bacci et al. 2015). When millions of DNA strands can be sequenced in parallel and many hundreds can be allocated to each section target, there is not necessity to generate a single DNA sequence (Shokralla et al. 2014). An even better solution is to openly include the ambiguity in species delimitation in the analysis, but in many cases, we need to increase our knowledge of taxonomy and evolution to do this precisely (Ryberg 2015). Our findings demonstrate the utility of Bayesian species delimitation methods and suggest that, wider application of these techniques will readily uncover new species in other cryptic fungal lineages. Preferably when two members of the same fungal species are verified, they should produce the same band pattern. Organisms of the similar genera but dissimilar species will share some bands, but produce a different overall pattern of bands. Organisms of two different genera would be projected to produce meaningfully different band patterns. Bands that fall within the same bin are called a match. The same DNA sample will not always generate the same band patterns, as small modifications to the amplification conditions will affect the resultant band pattern (Linacre, A, Cole & Lee 2002). The most promising candidate segment of DNA barcode is recognised ITS.

Conversely, a population genetic method with the analysis of the pattern of genetic inconsistency, using high-resolution molecular markers allows inferences

about the incident of recombination in the life history of the pathogen (Abadio et al. 2012). The smallest aggregation of populations with a mutual lineage shares single diagnosable phenotypic characters (Taylor et al. 2000). It is not necessarily right that, one “Phylotype” or “Operational Taxonomic Unit” or “Sequence Type” produced from an environmental sample is representative of a single organism (Jeewon & Hyde 2007). Undoubtedly, type specimens, especially holotypes, are the most important specimens for solving taxonomic issues (Hosaka, K & Uno 2013). A pure DNA sample of a single fungus or an amplified segment of that DNA could serve as type material. Otherwise, a duplicate of the sequence trace file could be selected as the type (Hibbett, DS et al. 2011).

Type statues voucher specimen / culture, country of origin and host / substrate of collection are three important factors for ecological investigation. The combination of three examined sequences produces the best-resolved phylogeny and the highest number of strongly supported clades. The results have become increasingly valuable in advancing knowledge about the Hypocreales in the use of molecular data to determine the relationships between taxa. The phylogenetic analyses using three markers resolved the problem of classification and showed the satisfactory level of neighbouring. MUMi is another distance-type index that is based on maximal unique precise matches shared by two genomes. The term Overall Genome Relatedness Indices (OGRI), utilize whole genome sequences, but not individual gene sequences or a set of sequences (Chun & Rainey 2014).

Genome BLAST distance phylogeny (GBDP) is a distance-type genome relatedness index. Mega-blast (Blast Software tool) and Molecular Evolutionary Genetics Analysis (MEGA) are two most important software that analyse the sequenced data.

A novel species can be recognized using the polyphasic tactic, in which we consider multi-dimensional features of organisms containing phenotypic and genotypic traits. In this process, genotypic categorization is a vital module in labelling species, as genetic information sheds light on evolutionary relationships between diverse heredities. Morphological and molecular features of a novel isolate justify its description as a new species. Closely related sister group can be

visualised at the base of each branch. The high similarity coefficient observed indicates that the two species are closely related. All the specimens are mapped onto a current phylogeny, based on DNA sequencing data. The longer the branches in tree means the more differentiation. Indeed, we reconstructed and recreated a multigene phylogeny for *Psilocybe*, using three nuclear markers.

The degree of similarities / differences of sequence types obtained from environmental samples also poses a problem. It is usually presumed that, for bacteria, >97% sequence identity can be regarded as different species. The sequences types cannot be confidently assigned to any particular genus or family, are referred to novel taxa or lineages. This is partially because DNA fingerprinting techniques do not provide any actual quantitative data regarding community function. It is likewise far easier to generate a putative uncultured sequence than to understand its biological importance from an applied perspective. Most of the molecular techniques involved, do not discriminate between active and inactive phases. This hampers an appropriate interpretation of the genetic / phylogenetic diversity with respect to ecology and function. The continued struggles to develop fungal database quality will moreover advance fungal ITS identification (Dannemiller et al. 2014).

13 Conclusion

Psilocybe mushrooms may produce psilocybin, psilocin and their by-products, although the concentrations produced range from non-existent to high. Most of the supposed psychoactive species are counted in the presently classified genus *Psilocybe* as described by (Guzmán, P et al. 1995) (Rossato et al. 2009). Although the genus name is based on the chemical found in some members of this genus, many of these species have not been chemically investigated, even though they are considered to be neurotropic owing to the 'bluing' of the basidiomes when bruised or damaged. Typically, the bluing colour is a manifestation of the oxidative response of the key toxins elaborated — psilocybin and psilocin. The study of molecular systematics of the larger group of psilocybin mushrooms supports separation of taxa from within the present genus *Psilocybe* (Rossato et al. 2009).

Psilocybe belongs to the much larger Agaric family, Strophariaceae which contain both highly chemically active species such as Death Caps (*Amanita phalloides*) as well as the most economic food species, the common edible mushroom (*Agaricus bisporus*) (Boekhout, Teun et al. 2002). Species of *Psilocybe* are a widely found genus and consists of approximately 150 potentially chemically active species, amongst 227 taxa found worldwide. There has been a resurgence of study on these types of mushrooms in various countries, most notably, Japan, Mexico, Brazil, Nepal and Thailand (Matsushima et al. 2009) (Guzmán, G et al. 2012) (Rossato et al. 2009) (Guzmán, G 2008) (Guzmán, G & Kasuya 2004).

John Hopkins Institute has been working on dosage levels of isolated and purified psilocybin to achieve maximum benefit in reducing fear and anxiety in cancer patients (Johns Hopkins University Psilocybin and Cancer) (http://www.heffter.org/research-jhuc.htm).

The main chemical found in potentially medically useful mushrooms is psilocybin. This naturally occurring compound is found in a wide variety of mushrooms, including the members of the genera *Psilocybe*, *Conocybe*, *Gymnopilus*, *Panaeolus*, and *Stropharia*. Uses of the chemical psilocybin have been

documented and range from minor, whole *Psilocybe* being used for weak fever, cold and toothache (Schultes, Hofmann & Rätsch 2001) to more profound, where the chemical is one of a small group of chemicals capable of passing through the blood-brain barrier and having intense effect on the principal nervous system (neuro-transmitter receptors).

Interestingly, psilocybin and psilocin have shown therapeutic success for obsessive –compulsive disorder (OCD), which is a notoriously hard-to- treat nervous illnesses (Matsushima et al. 2009). The use of psilocybin containing mushrooms is in many instances dismissed as a frivolous and indulgent pursuit of 'counterculture types' until Albert Hofmann (the famous Swiss natural products chemist) succeeded in isolating the active tryptamine alkaloid, psilocybin from these mushrooms (Grob, Bossis & Griffiths 2013).

Physiologically, psilocybin modifies brain concentrations of indole compounds as well as serotonin and therefore alters brain chemistry. Psilocybin is similar to morphine-6-glucuronide to a high degree because of the existence of a phenolic and tertiary amino group compounds (Sticht & Käferstein 2000). Additionally, psilocybin and psilocin are comparable in structure to serotonin (the neurotransmission substances in the brain) (tryptamine), an intracerebral neurotransmitter and act on serotonin receptors in the brain (Matsushima et al. 2009) (Schultes, Hofmann & Rätsch 2001).

The present study shows that the phenotypes of *Psilocybe* and morphologically similar genera have multiple evolutionary origins. Therefore, many of the morphological characters currently used to delimit the genera do not appear to be phylogenetically significant. Although morphological characters have been shown to be able to distinguish the culture of different genera of the isolated fungi, it is difficult to differentiate between the 96 *Psilocybe* species examined in this study on the basis of the colony morphology and hyphal characters. Three DNA regions are assessed as potential DNA barcodes for fungi, the second largest kingdom of eukaryotic. Results of molecular studies of this genus are useful in determining their suprageneric affinities. The evolutionary history is concluded with the help of Maximum-Minimum Likelihood method (Borovička, Rockefeller &

Werner 2012). The main inference proves that herbarium samples under one name should be categorised in group.

Ideally, molecular conclusions should be based on genetic data from liberated loci. We recommend the mutual use of both ribosomal and mitochondrial genes.

Usually, species diagnoses are based primarily on morphological characteristics displayed. Many new genera often defined by one easily determined character such as ascospore septation or colour. Some genera tentatively placed in, could not be conclusively included in the order because of the lack of definitive morphological characteristics. Uniting morphological and molecular data is obviously the best tactic to make progress in the study of mushroom identification rather than uniform morphology where limited characters are obtainable for morphological study (Hussein et al. 2014).

Good correlations are obtained between the two methods. Combination of morphological and molecular identification do not allow unambiguous species level identification for all fungal samples studied. Most of these studies rely on chemical analysis of ‘known species or the presently countless numbers of undescribed or recognised species. It is well known that there are potentially many new 'species' of *Psilocybe* in Australia and hypothetically several segregate genera. There may be species presently not catalogued or described that occur in Australia that have properties which are superior to those species already described and characterised. This study allows for a direct comparison with species already chemically profiled and identify potential candidates for further investigation and study. It also allows for a ready and systematic means of identification of described and presently undescribed entities in Australia. The methods developed and morphology / genetic 'fingerprints' identified in this study allow future researchers to quickly and easily identify taxa that are unknown to them. Importantly, identification of previously unknown taxa and clearer elucidation of existing taxa of *Psilocybe* and closely related species contribute to the overall documentation of the naturally occurring and introduced species of members of the Fungal Kingdom in Australia. I suggest that the Next Gene Sequencing technique is a reliable assay to distinguish the fungi. 96 species from the genus *Psilocybe* are characterized morphologic and molecularly. Some

doubts emerged from the genetics studies with respect to the basic differences that are required for strain to be considered different species. Mixed method has been useful for the identification and classification taxonomy of *Psilocybe* fungi. Sequenced-based approaches allow for a partial solution to the problem as much as they provide an opportunity to identify fungi.

Next Generation Sequencing is of countless worth because of its protocol simplicity, significantly reduced cost per barcode read, faster throughout and added information content.

The NGS-based DNA barcoding methodology as presented in our analysis is capable of recovering sequence variant in amplicons achieved from each specimen (Shokralla et al. 2014). Next-Generation Sequencing strategies offer meaningfully lower charge per base as compared to Sanger sequencing (Zoll et al. 2016). The best and cleanest assembly and least informative is LSU, between all three amplified regions. This study of the taxonomy of fungi in the genus *Psilocybe* is the first step in understanding the limits of this genus and in the identification of potential taxa within the genus in Australia and represents first-of-its-type research. The combination of two identification techniques allows for a high degree of resolution, to the genotype or even molecular individual level, and as such represents a major advance in taxonomic identification in fungi. The very specific identification of taxa using the combined methods employed in this research, lead to identification of potentially useful taxa with specific medicinal properties and the more rapid identification and refinement of possible pharmacological use. In the conclusion, we reemphasize that single locus data only should simply be used to deliver an introductory perception of species boundaries and not as the single sign in species restrictions (Leavitt, Moreau & Thorsten Lumbsch 2015).

The concatenate of three regions with 1792 sequence length and 56.9 % of the identicalness rates shows the support of sameness clustering of three regions. Also, the whole ITS region with 1184 sequence length and 57.9% identical rates proves the same idea.

In fact, Heterozygosity and Polymorphism is the main reason of differentiation between dissimilar obtained sequences. In practice assuming the most proper

level of population assembly using Bayesian clustering algorithms remains challenging. In this project we highlight the need for the reliability of DNA sequences deposited in public databases, as recently also suggested by several Authors.

14 Summary

This study contributes the first documentation of full description of the *Psilocybe* species supported by both morphological and phylogenetic data. Our results provide a better phylogenetic interpretation of morphological characters and their utility in determining generic delineations. Thus, it could be concluded that, these regions alone don't represent a universal diagnostic tool for the fungal species.

Both methods (morphology and genetic) found strong support for 96 species of *Psilocybe*, supporting species boundaries reflected in ITS divergence. Understanding the differences between morphological deviation within a species and among thoroughly related groups is crucial to identifying analytic characters, required for establishing precise phenotype based taxonomic boundaries. Application of molecular techniques assist us to iron out taxonomic confusion about *Psilocybe* group.

15 Perspectives and Recommendations

The study may serve as baseline information for further studies on the taxonomy of other genera of this mushroom. Further investigation with more taxa may help to conclusively resolve its phylogenies. It might be that inclusion of more sequences from different genes and maximum parsimony and Bayesian analyses will reflect and clarify more evolutionary and taxonomic issues. Additional studies, including comprehensive morphological and molecular studies of type material, are needed. It is clear that additional studies on this genus are needed in order to describe the extant diversity and gain insight into the evolution and biogeography of the genus. More research is needed to identify the active compounds. Here, we offer advice concerning the 'best molecular practice' for fungal taxonomy and systematic studies, in an attempt to aid unite the field and provide a solid foundation to support future work (Blasco-Costa et al. 2016).

Sequencing complete mitochondrial genomes is probably the next step beyond traditional DNA barcoding. Further studies are needed to determine how well suited the coalescence model is to model the evolution of the ITS region.

16 Supplementary Information

List of hallucinogens: LSD, PSILOCYBIN (MAGIC MUSHROOMS), DMT (DIMETHYLTRYPTAMINE), DATURA, MESCALINE (HTTP://WWW.VIC.GOV.AU/DRUGS & POISONS REGULATION IN VICTORIA) ( ) ( ) ( ) (http://www.vic.gov.au/Drugs & Poisons Regulation in Victoria) (A quick guide to drugs & alcohol). In contrast many countries have some level of rule or prevention of psilocybin mushrooms (for example, the US Psychotropic Substances Act, the UK Misuse of Drugs Act 1971, and the Canadian Controlled Drugs and Substances Act). The ban of psilocybin mushrooms has come under criticism because mushrooms are well-thought-out soft drugs with a little possible for misuse (HTTP://WWW.VIC.GOV.AU/DRUGS & POISONS REGULATION IN VICTORIA).

The Barcode of Life Data-Systems (BOLD) and GenBank are the main public repositories of DNA barcode sequences the identification systems (IDS) search algorithm

Index Fungorum (http:// www.indexfungorum.org/)

UNITE (https://unite.ut.ee/index.php) Australia Atlas (http://spatial.ala.org.au/?ss=17F347BB16396B55F9B0A7864949A952)

Australian Virtual Herbarium website (http://avh.chah.org.au/)

'Australasian Mycological Society'

(http://www.australasianmycologicalsociety.com/)

(http://molbiol-tools.ca/molecular_biology_freeware.htm)

(http://sequenceconversion.bugaco.com/converter/biology/sequences/index.php) (Useful to be able to convert different formats)

DNA sequence alignment is possible by these software; Bio-edit, Muscle, CHROMA, Geneious (EXPENSIVE but good)

Data analysis is possible by these software; PAUP*, MEGA, Phylip, MrBayes,

FigTree (program to visualize trees from MrBayes and other) BOLD=Life Database

Codon-code aligner is a commercial application for DNA sequence assembly, sequence alignment, and editing.

Several databases; (GenBank, EMBL, and DDBJ)

The GenBank (NCBI: http://www.ncbi.nlm.nih.gov/genbank/) database

MycoBank (an online database documenting new mycological names)

Index Fungorum (the global fungal nomenclature)

MAAR-Jam database (a web-based database containing Glomeromycota DNA sequence data)

International code of botanical Nomenclature (ICBN); the set of rules and recommendations dealing with the formal botanical names that are given to plants.

Protocols:

10048309.pdf

11306016.pdf

GeneRuler.pdf

Genomic DNA Extraction from Tissue of Plant.pdf

HighSensitivityDNAKit.pdf

KAPA-HiFi-HotStart-ReadyMix-PCR-Kit.pdf

KAPA-Lib-Quant-ILMN.pdf

Qubit-dsDNA-HS-Assay.pdf

17 Safety

The potential toxic elements of *Psilocybe* are well known and addressed as controlled substances under legislation. Appropriate Risk Assessments is carried out in association with established organisations (Royal Botanic Gardens Melbourne / avh.chah.org.au/) that have established protocols. Safe handling techniques for toxic substances are well established and is implemented here. Above all else, protection from ingestion of fungi or fungal by-products is of upmost importance. Drugs and poisons, when used appropriately, are of countless benefit, for instance in managing disease, creating valuable household products and reducing household pests. If not used correctly, they can harm general public.

State and Federal government have formulated strict regulations to governor their use, handling and production (www.parliament.vic.gov.au/.../9111-drugspoisons-and-controlledsubst...). The methods, by default are followed and implemented. In accordance with requirements of Victoria University and State and National Laws the appropriate precautions and legal requirements have been addressed.

- Preliminary Risk Assessments have been prepared and are appended.
- Additional Risk Assessments is prepared as required.
- The Poisons Control Plan and permits for drug storage at Victoria University have been updated for Footscray Park Campus and all requirements regarding the safe storage and handling of materials identified have been taken consideration and addressed.
- A requirement under the Poisons Control Plan for a specifically designed Drug Safe has been addressed and installed.

Data in this project is classified as sensitive data and is well protected. The use of R: drive with password protection goes part of the way to ensuring that data is only accessed by a limited number of people. These data protections should limit any misuse or abuse of this project as much as possible.

18 Conflict of Interest

The author declares that there is no conflict of interest related to this study.

19 Budget

Appendices 1, Budget 1.xlsx

Appendices 2, Budget 2.xlsx

20 Timeline

Appendices 3, TimeLine.xlsx

21 Reference List

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