PHYLOGENETIC AND MYCOCHEMICAL CHARACTERIZATION OF *TRAMETES* SPECIES FROM NORTHERN NAMIBIA.

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ABSTRACT

The objectives of this study were to confirm for the first time, the identity of *Trametes* mushrooms from Northern Namibia and then to generate information on local uses and bioactive compounds of these *Trametes* species. To achieve this, samples were collected from Ohangwena, Omusati, Oshikoto and Oshana regions and identified as *Trametes* species based on morphological features. These were a basidiocarp with concentric zones, brown, tan or grey colour and pores from 2-3 pores/mm to pores slightly bigger than 1 mm in diameter and lack of a pileus. TLC visualization reagents were used to identify compound groups in the hot water and organic extracts. The resulting chromatogram showed nine compounds separated in total and indicated presence of flavonoids, alkaloids and triterpene saponins. The disc diffusion method was used in bioassays of the extracts dissolved in DMSO and water. The highest zone of inhibition was 12 ± 1 mm by the DCM extract on *Escherichia coli.* The lowest inhibition observed was 9.7 ± 2.08 mm by the DCM extract on *Bacillus subtilis*. Interestingly, no inhibition was observed for the methanol and HWE extracts. These results differ from previous studies which reported that all organic extracts of *Trametes* were active against *B. subtilis*. A questionnaire was used to obtain indigenous knowledge of *Trametes* mushrooms in Northern Namibia. Questionnaire data analysed in SPSS revealed that 83.9 % of respondents in this study knew *Trametes* mushroom but only 70.4 % used it for medicinal purpose. The most common medicinal use of this mushroom was to calm bereaved people crying hysterically at burials and also to treat lung disease in cattle. The

results from this study are novel since they have not been reported in literature before. Finally, fungal DNA was extracted and PCR performed with ITS1/ITS2 primers before sequencing. BLAST searches revealed that specimen collected were 99 % identical to *Trametes polyzona*. A phylogenetic tree was reconstructed using the Neighbour Joining method in MEGA version 6. The specimens collected from Northern Namibia were distributed in 6 clades mainly alongside *Trametes* species, *Truncospora* and more distantly *Coriolopsis* species. These results are consistent with previous works which agree that there is a close relationship between the genera *Trametes*, *Coriolopsis* (*polyzona*) and *Pycnoporus*.

Keywords: *Trametes* species, medicinal, mushroom, phylogenetic, TLC, ethnomycology

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DECLARATION

I, Isabella Shaningika Etuhole Ueitele hereby declare that this study is a true reflection of my own work and that this thesis or part thereof has not been submitted to any other institute of higher learning for any degree.

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DEDICATION

I dedicate this thesis to Abba, my heavenly Father, who has been there from the very beginning. I love you, Lord. I also dedicated this work to my parents, Mr. Izak Ueitele and Mrs. Natangue Tangeni Ueitele, and to uncle Shafi. Thank you for always giving me and my siblings the best. May God richly reward and favour you. May you see the goodness of the Lord in the land of the living.

ABBREVIATIONS

$AlCl_3$	Aluminium chloride
ATCC	America Type Culture Collection
BLAST	Basic Local Algorithm Search Tool
bp	Base pairs
CWDE	Cell wall degrading enzymes
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GC-MS	Gas chromatography- Mass spectrometry
HIV	Human Immuno-deficiency Virus
HPLC	High Pressure Liquid Chromatography
HWE	Hot Water Extract
ITS	Internal Transcribed Spacer
KOH	Potassium Hydroxide

LAK	Lymphokine Activated Killer
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
Mn	Manganese
NGSTP	Namibia Government Scholarship and Training Program
nLSU	Nuclear Large Subunit
NMR	Nuclear magnetic resonance
NPC	Namibia Planning Commission
NSA	Namibia Statistics Agency
PCR	Polymerase Chain Reaction
PSK	Polysaccharide- Krestin
PSP	Polysaccharide Peptide
rDNA	Ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPB	Riboflavin Binding Protein

rpm	Revolutions per minute
SPSS	Statistical Package for Social Sciences
SSU	Small subunit
TBE	Tris Borate EDTA
TEF	Transcription elongation (enhancement) factor
TLC	Thin layer chromatography
UNAM	University of Namibia
UV	Ultraviolet
ZERI	Zero Emissions Research Initiative

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PUBLICATIONS

The following articles have been presented in conference proceedings or prepared for publication in peer reviewed journals. They are based on the work carried out in this thesis.

Conference Papers and Posters

1. **Ueitele, I. S. E.,** Chimwamurombe, P. M., Louw, S., and Kadhila-Muandingi, N. P., (2015). Mycochemical and Antimicrobial Screening of indigenous *Trametes* species from Northern Namibia. Paper presented at the 3rd Annual Science Research Conference, 18-19 November 2015. University of Namibia. Windhoek, Namibia.

2. **Ueitele, I. S. E.,** Chimwamurombe, P. M., and Kadhila-Muandingi, N. P., (2015). Molecular phylogeny of *Trametes* and related genera from Northern Namibia. Poster presented at the International Symposium on Methods for Studying Drug Metabolism and Transport, and African Traditional Medicines, 23-25 November 2015. Pretoria, South Africa.

3. **Ueitele**, **I. S. E.,** Chimwamurombe, P. M., Louw, S., and Kadhila-Muandingi, N. P. 2014. Ethnomycology of Indigenous *Hexagonia* species in Northern Namibia.

2nd Annual Science Research Conference, 30-31 October 2014. University of Namibia. Windhoek, Namibia.

Journal Articles

1. **Ueitele, I. S. E.,** Chimwamurombe, P. M. and Kadhila-Muandingi, N. P., (2016). Molecular phylogeny of *Trametes* and related genera from Northern Namibia. Manuscript in preparation.

2. **Ueitele, I. S. E.,** Louw, S., Chimwamurombe, P. M., and Kadhila-Muandingi, N. P., (2016). Mycochemical and antimicrobial screening of indigenous *Trametes* species from Northern Namibia. Manuscript in preparation.

3. **Ueitele, I. S. E.,** Chimwamurombe, P. M., and Kadhila-Muandingi, N. P., (2016). Ethnomycology of *Trametes* mushrooms in Northern Namibia. Manuscript submitted for publication.

CHAPTER 1: Introduction

1.5 General Introduction

Several species of fungi which belong to the Basidiomycetes and Ascomycetes produce fleshy fruit bodies called mushrooms (Krishna et al., 2015). They grow in a wide range of habitats and ecosystems, appearing in all seasons, especially rainy weather when abundant organic matter is available and more easily degradable (Jha & Tripathi, 2012a; Afiukwa et al., 2013). Although not a taxonomic term, mushrooms are widely described as macro fungi with fruiting body visible to the naked eye, and which can be hypogeous or epigeous (Jha & Tripathi, 2012a). Mushrooms are hypogeous when they appear below the ground and epigeous when they grow above ground (Afiukwa et al., 2013). The fruit body is a reproductive organ of the fungus in which spores are produced and released (Falandysz & Borovička, 2013).

It is not an easy task to distinguish edible mushrooms from medicinal mushrooms because some may be considered both edible and medicinal (Valverde, Hernàndez-Pérez & Paredes-López, 2015) for example *Lentinula edodes*. Mushrooms can also be edible or inedible (Falandysz & Borovička, 2013) as well as inedible but medicinal (Krishna et al., 2015) for example *Trametes* and *Ganoderma*. They are heterotrophic, saprophyte or parasite feeders (Falandysz & Borovička, 2013) which feed by breaking down organic matter into essential nutrients. The lignocellulosic materials from which macro fungi

grow and obtain their nutrition are called substrates. Macro fungi are not only visually appealing, they also play a significant role in the daily lives of human beings along with their use in industry, agriculture, medicine and many other ways (Jha & Tripathi, 2012a; Halpern, 2007).

The mushrooms from the genus *Trametes* Fr. Have pileate sessile basidiocarps, di or trimitic hyphae systems, smooth non-dextrinoid and non-amyloid spores. They have a highly variable hymenium and cause white rot. There are close to 60 known *Trametes* species (Oyetayo, 2014; Knežević et al., 2015) which grow in almost every forest ecosystem and grow frequently on many hardwoods. *Trametes* are wood decomposers and have enormous potential for bioremediation and biodegradation, economic and ecological importance as well. This mushroom is closely related to *Coriolopsis* Murril., *Lenzites* Fr. And *Pycnoporus* P. Karst. *Trametes elegans* is widespread in tropical and subtropical environments (Carlson, Justo, & Hibbet, 2014; Oyetayo, 2014). *Lenzites*, *Trametes*, *Coriolopsis* and *Pycnoporus* species are common in the tropical rain forest of Nigeria (Oyetayo, 2011).

Trametes versicolor is possibly the most important species in this genus and is well recognized by food and pharmaceutical researchers (Zmitrovich, Ezhov & Wasser, 2012). While *Trametes* has been used in Traditional Chinese medicine for detoxing, energy boosting, improving liver and spleen function in the form of tea, modern

medicine mainly uses this mushroom to treat various types of cancers, chronic hepatitis, rheumatoid arthritis, and infections of the respiratory, urinary, and digestive tracts (Oyetayo, 2014; Knežević et al., 2015). The polysaccharides are the main contributors to its medicinal quality (Valverde et al., 2015; Chen & Li, 2015). Kerstin (PSK) and Yunzhi are commercial products produced from *T. versicolor* and are used as immune modulators in adjuvant therapy to treat cancer (Knežević et al., 2015).

Compared to other African countries, Namibia is behind and needs to emulate other countries such as Nigeria whose researchers over the last five years have been focusing their research on investigating the medicinal use of mushrooms (Oyetayo, 2011). In this study the Namibian indigenous *Trametes* species were identified and characterized using the Internal Transcribed Spacer (ITS) region of genomic DNA. The types of mycochemicals present were determined by using Thin Layer Chromatography (TLC). The main objective was to generate information on genetic diversity, uses and bioactive mycochemical compounds of *Trametes* species from Northern Namibia.

1.2 Problem Statement and Justification

For the past decade researchers in Namibia have developed interest to study the indigenous mushrooms of Namibia. Recent studies by Kadhila-Muandingi and Chimwamurombe (2012) and Ekandjo and Chimwamurombe (2012) have focused mostly on medicinal mushrooms, specifically *Ganoderma* species. The problem remains that most Basidiomycetes in Namibia need to be explored in order to document and preserve the Namibian mushrooms biota and biodiversity, especially considering that Africa's rapidly increasing population, combined with deforestation, will likely cause endangerment to the mushroom biota (Chang & Mshigeni, 2004). This is especially important when taking into consideration that mushroom biota grows in association with the indigenous vegetation (Krishna et al., 2015).

Furthermore, the incorrect taxonomy of many medicinal mushrooms jeopardizes the validity of current and future investigations of these mushrooms and their derivatives (Zmitrovich et al., 2012; Wasser, 2011). The use of general names like Turkey tail (*Trametes versicolor*) and Ling Zhi or Reishi (*Ganoderma lucidum*) makes room for mistaken identity of specific species and type material (Wasser, 2011; Wasser, 2014). Therefore, there is a need for consistency in the identification of medicinal mushrooms like *Trametes* species to ensure that future investigations of their medicinal properties, composition and effectiveness are done on the right species. This will help to prevent the adulteration of medicinal mushroom products with counterfeit species such as *Stereum*

species for *Trametes* species and *Ganoderma* species for *Ganoderma lucidum*, which could lead to serious side effects like nephropathy, acute hepatitis, fever and even coma (Wasser, 2011; Wasser, 2014). It is on the background that this study will characterize indigenous *Trametes* species from Northern Namibia in order to generate information on its genetic diversity, uses and bioactive mycochemical compounds in order to start solving the problem created by lack of full information for utilization of native mycobiota.

1.3 Objectives of the Study

Emerging from the above stated problems, the objectives of the study were:

a. To construct the phylogeny of *Trametes* species in Northern Namibia.

b. To determine the different uses of *Trametes* species by the local people in Northern Namibia.

c. To determine the different types of mycochemicals present in *Trametes* species from Northern Namibia which may have potential medicinal properties.

1.4 Hypotheses of the Study

a. The *Trametes* species from Northern Namibia all belong to the main Trametoid clade.

b. The local people of Northern Namibia have different applications for *Trametes* species due to differences in culture and therefore ethnomycology.

c. *Trametes* species from Northern Namibia have varying alkaloids, anthraquinones and other mycochemicals due to differences in age of fruiting bodies and environmental factors such as water availability, substrates and temperature.

1.5 Relevance of the study

This was the first study in Namibia that was done on indigenous *Trametes* species to fill the gap on underutilized and poorly studied mushrooms in the country. Mapping out the genetic diversity, uses and mycochemical compounds of *Trametes* species will serve as a baseline study for the importance of this mushroom. Confirming the identity of

Namibian indigenous *Trametes* mushrooms will help to validate any future investigative studies on these species, especially for therapeutic use.

Furthermore, the results of this study could stimulate a wider interest in Namibia's mushrooms, leading to further research on the bioactive compounds which can be used in medical sciences and also build literature repository of the Namibian mushrooms. Lastly, the benefits of mushrooms, including *Trametes*, cannot be exhausted, ranging from the potential discovery of new drug scaffolds for drug discovery purposes to conserving the biodiversity of Namibia's mushroom habitats.

1.6 Limitation of the study

The distribution and geographical location of *Trametes* species in Northern Namibia was too large to fully explore the entire region. The results obtained in this study are therefore representative and do not necessarily depict the full picture of genetic diversity, antimicrobial content, ethnic knowledge and use of the mushroom as it would be beyond the scope of this work to have such a broad and costly study. Also, some of the potentially bioactive compounds may not always be present in sufficient quantities to permit full chemical characterization or bioactivity testing. This may be due to various factors such as environmental conditions, solvents used and specific mushroom species and isolates (Yamaç & Bilgili, 2006).

CHAPTER 2: Literature Review

2.1 Mushrooms

The Kingdom Fungi is one of the largest, most diverse and economically important group of organisms (Martin et al., 2011; Charya, 2015). Fungi are probably the most important decomposers of organic matter on earth. They display elaborate genetic and metabolic diversities which allow them to colonize a wide range of substrates in the most complex environments (Wisecaver, Slot & Rokas, 2014). This domain is divided into five basic phyla, namely the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota (Figure 1). The Fungi are an assorted group of unicellular and multicellular eukaryotic organisms such as yeasts, filamentous fungi, lichens and macrofungi including mushrooms and puffballs (Madigan, Martinko, Dunlap & Clark, 2009; Heitman, 2011). Although the Fungi are estimated at around 13.5 million species worldwide, only 31 503 have been described (Abdel-Azeem, 2010).

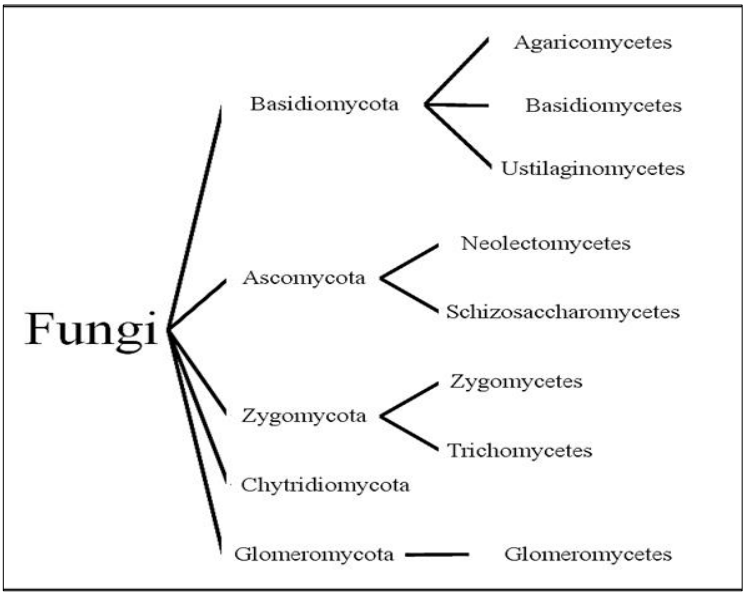

Figure 1. The Kingdom Fungi is divided into 5 phyla. Included in this figure are some of the classes found in each phylum.

Chytridiomycota were the earliest divergent lineage of fungi and are considered to have aquatic origins. Their motile flagellated spores, called zoospores, allow for movement and propagation in aquatic environments (James et al., 2006). Zygomycota are commonly referred to as soil fungi and a well-known species from this group is *Rhizopus stolonifera*. These fungi also cause food spoilage (Madigan et al., 2009). The phylum Glomeromycota is a small group of fungi but with major ecological significance (Kruger, Stockinger, Krüger & Schüβler, 2009). All members of this phylum have symbiotic associations with herbaceous and some woody plants. The fungal hyphae enter plant cell to produce swollen vesicles often called arbuscles. This phenomenon is known as endo or arbuscular mycorrhiza (Madigan et al., 2009). The Ascomycota are a

large and diverse group which ranges from single cell yeasts to filamentous molds. These fungi form symbiotic relations with cyanobacteria or green algae to form lichens and with plants to form ectomycorrhizae (Stajich et al., 2009; Madigan et al., 2009).

The phylum Basidiomycota, also known as the true fungi, contains mushrooms, puffballs, bracket fungi and ectomycchorhiza (Abdel-Azeem, 2010). Rusts and smuts, some of the world's most devastating plant pathogens also belong to this phylum (Abdel-Azeem, 2010). The phylum Basidiomycota contains some of the most important primary decomposers of dead organic matter, recycling and returning organic carbon back to the environment (Abdel-Azeem, 2010; Bass et al., 2007). Being heterotrophic saprophytic feeders, these fungi attach to a host and secrete enzymes into the host. The fungal enzymes break down the host complex polymers into simpler products which are more readily available to other organisms (Valentín et al., 2009; Jha & Tripathi, 2012b). Furthermore, as a result of their metabolism, fungi produce essential amino acids, vitamins, enzymes and other metabolites. These metabolites have been developed into therapeutic agents (Roberts, 2004) and used to produce some of the best antibiotics. Penicillin, Cephalosporin and Griseofluvin are excellent examples (Perlman, 1974; Wasser, 2011).

The Agaricomycetes are a class of mushroom forming Basidiomycota (Figure 1). This is a diverse group of fungi which constitutes close to 1/5 of all known fungi and all the edible mushrooms (Hibbett et al., 2014). They form diverse reproductive structures called fruiting bodies, which range from a simple crust to conspicuous mushrooms and puffballs (Hibbett, 2006). With an estimated 140 000 species of mushrooms on earth, only 22 000 have been described (Lindequist, Niedermeyer & Jülich, 2005). Mushrooms, rich in proteins and essential amino acids, minerals and vitamins are the perfect health food (Dundar, Acay & Yildiz, 2008). Apart from that, they are low in total fat content and have a high proportion of polyunsaturated fatty acids (72 % to 85 %) relative to total fat content, mostly due to their high linoleic acid content (Dundar et al., 2008). Mushroom proteins are inclined to be easily digested and their content ranges between 20 % and 40 % protein, better than many legume sources like soybeans and peanuts, and vegetables (Ziarati & Ghasemynezhad-Shanderman, 2014). What's more, mushroom proteins contain all the amino acids essential to the human diet and are especially rich in lysine and leucine, which are usually lacking in staple cereal foods (Dundar et al., 2008).

Apart from being a source of food and therapeutic agents, fungi also cause great economic losses in the Agricultural industry. Various fungal species such as *Aspergillus*

and *Alternaria* species infect crops, especially fruit and cereals. These fungal infestations cause spoilage during ripening in the fields as well as at storage and processing stages (Ibrahim & Rahma, 2009). Not only do these fungi cause reduction in the quality and quantity of the fruits, some species produce mycotoxins called aflatoxins, which are highly toxic to humans and animals when ingested (Ibrahim & Rahma, 2009; Petrović et al., 2013).

2.2 The Life Cycle of Mushrooms

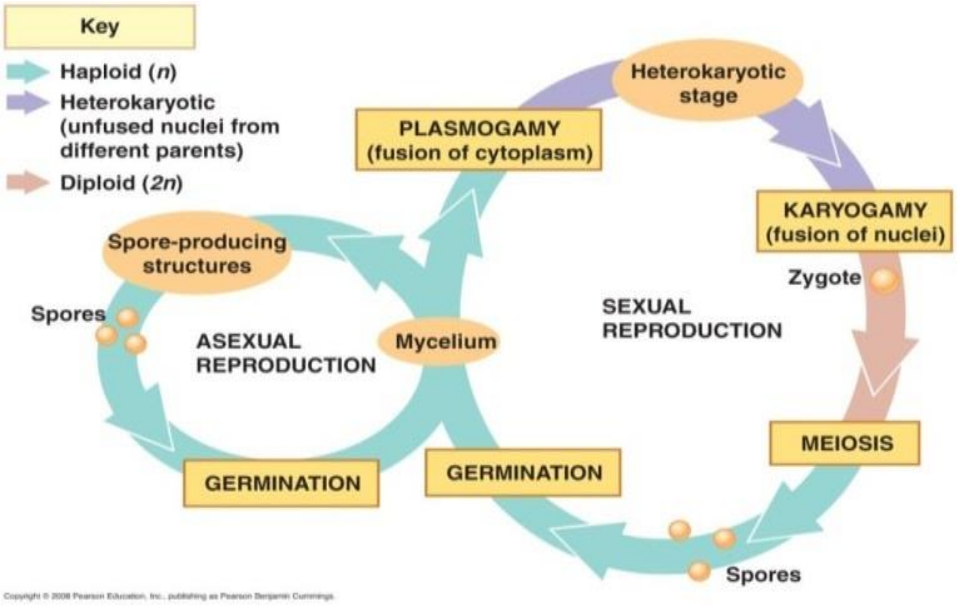

Figure 2. Life cycle of mushrooms (Campbell & Reece, 2005).

Fungi can reproduce both sexually and asexually (Figure 2). Mitospores are produced during asexual reproduction and meiospores during sexual reproduction. The two types of spores are produced by the same mycelium but they differ greatly in form and are easily recognized. In the life cycle, asexual reproduction happens first and may even be repeated frequently before sexual reproduction occurs (Casselton & Olesnicky, 1998). When the conditions are favorable, millions of spores are released and the spores germinate, sending out tiny threads called hyphae. These hyphae find other hyphae they are compatible to and fuse together to form a network of threads called a mycelium (Figure 2). The mycelium grows into the medium on which it is growing with other parts of it being aerial. The aerial hyphae produce haploid spores which germinate asexually (Casselton & Olesnicky, 1998; Ni, Feretzaki, Sun, Wang & Heitman, 2011).

This mycelium grows until it finds hyphae from another fungus. The two fungi will fuse and if they are sexually compatible exchange of genetic material occurs. When the compatible haploid nuclei are in the same hyphal tip the cycle enters a dikaryon rather than a diploid stage (Casselton & Olesnicky, 1998). This is an interesting phenomenon of Basidiomycetous fungi known as plasmogamy (Madigan et al., 2009). Favourable environmental conditions induce aggregation of the aerial dikaryotic hyphae, which eventually form a visible fruiting body. These develop into mature fruiting bodies and allow karyogamy and meiosis to occur in the basidia within the mature fruiting body (Figure 2). Nuclear fusion and meiosis occurs in the basidia and a zygote is formed before it eventually develops into a pinhead and grows into a mushroom. The mushroom

will release spores again and the cycle continues. The haploid and diploid phases alternate. The haploid phase, which produces the gametes, is initiated by meiosis. One will find that in most fungi, all structures except the zygote are haploid (Ohm et al, 2010; Casselton & Olesnicky, 1998).

There are some variations in the life cycles of different mushrooms (Ni et al., 2011). An example is mushrooms which only reproduce by sexual means and others only asexually while some are parasexual. Plasmogamy, karyogamy, heterokaryosis and meiosis are common in parasexual fungi (Cole, 1996).

2.3 Industrial application of *Trametes* species

Increasing development in agriculture and industry produces a huge amount of heavily degradable organic matter as a waste product. Most of the waste is burned or buried to get rid of it. This causes great harm to the environment (Dorado, Van Beek, Claassen & Sierra-Alvarez, 2001). About 97 % of this waste is lignin rich and if treated properly can be a good source of energy, provide nutrients to the soil which can be taken up by plants and conserve organic matter in the agroecosystem (Knežević, Milovanović, Stajić & Vukojević, 2013; Dorado et al, 2001). In order to get to the available energy, lignin has to be degraded first (Silva et al., 2014) and only few organism can break down lignin. In this view, the introduction of lignin degrading species seems to be a potential solution to this problem.

Fungi are generally cellulose and hemicellulose degraders but Basidiomycetes are the fungi largely responsible for lignin decomposition. Basidiomycetes are arranged in three decay groups based on the way they attack the wood; white, brown (Silva et al., 2014) and soft rot (Valentín et al., 2009; Børja, Alfredo, Filbakk & Fossdal, 2015). Brown rot fungi such as *Serpula lacrymans* and *Fomitopsis pinicola* can only digest lignin from their host and make the cellulose available to other saprobes. White rot fungi such as *Trametes* species and *Pleurotus ostreatus* can completely break down the cellulose, hemicellulose and lignin simultaneously (Dorado et al, 2001; Skrede et al., 2011). Even in nature they grow on woody substrates (Datta, Bettermann & Kirk, 1991; Machuca & Ferraz, 2001). White rot fungi are the most effective lignin degrading microorganisms and can completely convert lignin to carbon dioxide (Sun, Li, Yuan, Yan & Liu, 2011) and water (Have & Teunissen, 2001).

Three special enzymes are produced by these basidiomycetous fungi to break down the lignin, namely lignin peroxidase, manganese peroxidase and lacasses (Valentín et al., 2009). The lignin degrading ability also depends on the genera, species and even strain of the fungus (Silva et al., 2014). White rot fungi like those from the genus *Trametes* hydrolyze cellulose and hemicellulose to support mycelial growth, metabolism and to break down lignin. Hemicellulose is usually degraded faster than cellulose because it is simpler in structure and it provides nutrients for survival. As a result most of the plant material affected by white rot is cellulose rich (Sun et al, 2011).

Fungi also produce and secrete a host of other cell wall degrading enzymes (CWDE). These CWDE allow entry into plant cells so that saprophytic fungi can obtain nutrients from plant cells and for pathogenic fungi to infect and cause disease (King et al., 2011; Zhao, Liu, Wang & Xu, 2013; Kubicek, Starr & Glass, 2014). Glycoside hydrolases, polysaccharide lyases, carbohydrate esterases, glycosyl transferases and carbohydrate binding molecules have been isolated from Ascomycetes, Basidiomycetes, Zygomycetes and Chytridiomycetes. Although these enzymes have been detected in almost all fungi, it is notable that they are found in higher concentrations in plant pathogenic fungi (Zhao et al., 2013).

Through biodegradation, fungal species can be used to reduce lignin, thereby providing a larger surface area to make the cellulose accessible to its degraders (Knežević et al, 2013). Biodegradation is viable both environmentally and economically because it takes place under milder conditions, consumes less energy, is fairly easier to carry out and the equipment needed is much cheaper (Sun & Cheng, 2002). Lignin degradation not only provides carbon and energy but also allows the fungus access to the cellulose and hemicellulose. Cellulose and hemicellulose are broken down by cellulose and hemicellulose enzymes while lignin is broken down by peroxidase and laccase (Lekounougoua et al, 2008). Laccases are commonly produced by fungi such as *Trametes versicolor*, *Pleurotus ostreatus* and *Ganoderma lucidum* (Bodke, Senthilarasu & Raqhukumar, 2012). They play a huge physiological role in fungal growth and

development. They act as fungal virulence factors, pigments of fungal spores and in lignifying cell walls as well as causing white rot on wood. Laccases have commercial importance as they are used to make paper pulp, ethanol and to differentiate morphine from codeine. They are also employed in bioremediation to reduce the impact of industrial toxins on the environment (Dorado et al, 2001; Bodke et al., 2012).

The ability of white rots to degrade wood extractives has been investigated extensively in the field of paper pulp manufacturing (Paice, Reid, Archibald & Jurasek, 1993; Lekounougoua et al, 2008). When two *Trametes* species were cultivated on wheat straw, *T. pubescens* showed higher laccase activity and manganese peroxidase (MnP) production than *T. multicolor* although *T. multicolor* showed the highest protein content 10 days after cultivation. Their lignin degradation was also compared by cultivating on oak wood sawdust with lignin content equal to 171 mg/g. The degradation rate was 1.8 % in *T. pubescens* and a slightly higher 5.9 % in *T. multicolor*. The authors argued that there is a parallel between the level of enzyme activity and lignin loss. They further argued that Mn-oxidizing peroxidases might have been responsible for degrading the lignin despite the low laccase activity (Hofrichter, 2002). Oak sawdust is the natural substrate for *Trametes* species (Knežević et al, 2013).

Trametes species have shown significant potential to use agricultural waste as a substrate for producing ligninolytic enzymes such as laccases and Mn-dependent peroxidases. If studied properly, these species can lessen the burden of environmental

pollution and provide renewable and biodegradable energy sources. Previous studies have demonstrated that although not all, some species in this genus can use wheat straw as a substrate to produce ligninolytic enzymes and Mn-oxidizing peroxidases at varying ratios (Knežević et al, 2013).

Trametes laccases have also shown great ability to break down industrial dyes (Sharma, Shrivastava & Kuhadi, 2015) such as indigo carmine, phenol red and bromophenol blue at different rates, depending on the structural makeup of the dye (Moldes, Gallego, Couto & Sanrom´an, 2003). The ability of a fungus to decolorize type model dyes is one of the simplest methods used to evaluate the ligninolytic enzymes ability to break down aromatic compounds. *Trametes hirsuta* was cultivated in solid state and the extracellular enzymes were exposed to different dyes. *T. hirsuta* showed a high potential to degrade even azo dyes which are usually not discolored to a great extent. The laccase enzymes however did show selectivity to the type of dye structure (Moldes et al., 2003). Lastly, *Trametes trogii* is a worldwide distributed mushroom which also degrades and causes white rot on wood. It has been isolated from dead acacia wood in Northwest Tunisia (Zouari-Mechichi et al., 2006).

2.4 Medicinal Compounds of Mushrooms

With increasing incidences of multiple resistances of human pathogenic microorganisms due to the indiscriminate use of antibiotics, antibiotic resistance has become a global concern. This has led to a search for new antimicrobial agents from sources such as mushrooms and plants (Parekh & Chanda, 2007; Kothari, Shah, Gupta, Punjabi & Ranka, 2010). The Kingdom Fungi is well renowned for its contribution of bioactive metabolites for the development of medicine (Dembitsky, 2014). With their impressive metabolic diversity, Basidiomycetes open new avenues in biotechnology to produce many flavour and volatile molecules as well as enzymes (Dembitsky, 2014). Fungal metabolites have been useful in organic chemistry for years. In many cases, they were the building blocks that led to advances in developing methods for synthesis of lead compound analogues. The analogues usually have improved pharmacological properties. The natural product scaffolds are also recognised as superior structures because of their ability to be the basis for successful drug production or discovery (Qiu et al., 2014).

Traditional healing systems which use herbal remedies provide very important clues in the discovery of new antibiotics. This is evident with the amount of research focusing on validating the use of mushrooms and plants to manage and treat various conditions (Parekh & Chanda, 2007; Kothari et al., 2010). Molefe-Khamanga, Mooketsi, Matsabisa and Kensley (2012) have also reported that many researchers are dedicated to

investigating the phytochemical quality of plants which have traditional knowledge associated with them. In fact numerous studies maintain that traditional remedies have provided antibiotic compounds to resistant strains of bacteria (Romero et al., 2005; Menghani, Ohja, Negi, Agarwal and Pareek, 2011). Traditional healing systems also aid in leading to the synthesis of more potent drugs with reduced toxicity from the herbal remedies (Parekh & Chanda, 2007). This makes traditional remedies very attractive as possible therapeutic agents, especially when considering their availability and relative affordability compared to the 'modern' medicines, especially in Sub-Saharan Africa (Molefe-Khamanga et al., 2012).

Trametes, Ganoderma, Tremella, Lentinus, Grifola and Cordyceps are some of the genera of Basidiomycetes strongly recommended for treating a variety of illnesses (Oyetayo, 2014). Wild and cultivated mushrooms make a significant contribution to modern medicine because they offer an unlimited source of polysaccharides with antitumor and immune-stimulating properties (Wasser, 2002; Chen & Li, 2015) available from fruit bodies, cultured mycelium or culture broth (Wasser, 2002). Secondary metabolites are a group of organic compounds which are not involved directly in the growth and development of the mushroom but play a vital role in defense against pathogens and predators (Kothari et al., 2010). These metabolites give mushrooms their medicinal properties and are what make mushrooms beneficial to the immune system of humans and animals (Halpern, 2007; Pereira, Barros, Martins & Ferreira, 2012). An interesting example is that of mushrooms in the order Russulaceae.

These mushrooms contain biologically inactive fatty acid esters of a sesquiterpene alcohol which are transformed when tissues are damaged. It was reported that this transformation takes place in a matter of hours, sometimes seconds. The sesquiterpenes are transformed into ‘second generation’ metabolites which range from tasteless to bitter or acrid compounds. It is suggested that the resulting bitter sesquiterpenes in injured fruit bodies is a form of a chemical defense system, where the fruiting bodies are protected from microorganisms and predators (Malagòn et al., 2014).

Although not all secondary metabolites exhibit this defense system (Kothari et al., 2010), those that are bioactive are categorized into groups such as phenolics, flavonoids, xanthones, coumarins, tannins and terpenoids. Each mushroom type produces a specific set of metabolites capable of dealing with the set of microbes that coexist in that specific environment (Colak, Faiz & Sesli, 2009). It is therefore expected that one mushroom type would possess bioactivity different from other mushroom types. To demonstrate this, the *Amanita* species produce secondary metabolites called amanitins, which are permanently present in the mushroom. Also, compounds isolated from *R. nobilis* differentiate them with respect to other *Russula* species investigated (Malagòn et al., 2014). *T. versicolor* is widely recognized because it possesses β-glucans known as Krestin which have strong anticancer properties. Antioxidant activity has also been reported in this powerful mushroom (Oyetayo, 2014).

What's more, evolutionists have pointed out that the Kingdom Fungi is more closely related to animals than to plants (Abdel-Azeem, 2010; Heitman, 2011). They are closely aligned in one of the six to eight super groups of eukaryotes, the opisthokonts (Heitman, 2011). Consequently, since they are closely aligned, they share the same pathogens (Abdel-Azeem, 2010; Leliebre-Lara, García, Nogueiras & Monzote, 2015). It is no wonder that almost all the useful antibiotics to humans have come from the fungi.

2.4.1 Phenolic compounds

Phenols are compounds which consist of an aromatic ring bearing one or more hydroxyl groups (Figure 3). Thousands of phenolic compounds with high molecular diversity are known. The total phenolic contents recorded for mushrooms such as *Trametes versicolor* and *Fomes fomentarius* range from 4.07 to 147.78 ± 5.21 mg/ml (total phenolic) and 8.13 to 138.80 ± 6.51 mg/ml (total flavonoid) (Abugria & McElhenney, 2013). Literature shows that although present, phenolic compound concentrations in mushrooms are quite low compared to plants (Ribeiro et al., 2008). One must bear in mind however, that the extract yield does not always associate with the total phenolic content of wild mushrooms. However, it can be influenced by the types of phenolic compounds present and the extractability of the solvent used in the preparation (Wong & Chye, 2009). These secondary metabolites are classified according to the number of carbon atoms and their structure or according to their origin of synthesis i.e. chemical or natural pathways in biosynthesis. Based on the number of phenol units in a molecule,

they are classified as simple phenols or polyphenols (Soto, Moure, Dominguez & Parajó, 2011; Khoddami, Wilkes & Roberts, 2013).

Figure 3. Structures of two phenolic compounds, Fumigatin, a quinone (Left) and Thymol, a monoterpene (Right).

Phenolic compounds exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, hepato-protective, anti-thrombotic, antiviral, anticarcinogenic and vasodilatory. Many of these biological functions are attributed to the phenols' free radical scavenging and antioxidant activity (Barros, Venturini, Baptista, Estevinho & Ferreira, 2008). It is believed that their hydroxyl groups may contribute directly to their antioxidative action (Wong & Chye, 2009). Phenols provide antioxidant activity to prevent heart disease, reduce inflammation, and lessen the possibility of getting cancer as well as reducing the rate of mutagenesis in human cells (Soto et al, 2011; Khoddami et al., 2013).

Phenol compounds have attractive colours and they are used frequently as natural colourants. They have excellent properties used in food preservation when food products containing lipids and its associated foods are supplemented with phenols to inhibit lipid peroxidation and to lengthen the shelf life of products (Soto et al., 2011; Abugria & McElhenney, 2013). Flavonoids account for more than half of the different phenolic compounds (Balasundram, Sundram & Samman, 2006). Flavonoids are polyphenols which help to reduce oxidative stress and induce growth inhibition and cell death of cancer cells (Flora, 2009). More examples of phenolic compounds include phenolic acids, tannins, coumarins, curcuminoids, quinones such as fumigatin, lignins, lignans, benzoic and cinnamic acid (Huang, Cai & Zhang, 2010; Khoddami et al., 2013)

2.4.2 Alkaloids

Alkaloid compounds occur in nature as a class of nitrogenous organic compounds (Figure 4) in plants, fungi and bacteria (Ikan, 1991; Dembitsky, 2014, Qiu et al., 2014). Most alkaloids are basic, colourless and crystalline compounds which act as a reserve material for protein synthesis in the organism. Their bitter taste offer protection to the plant and fungi from animal and insect predators (Ikan, 1991). The alkaloids have not only been used in medicine to treat humans and animals but poisonous types have also been used in hunting and execution (Amirkia & Heinrich, 2014).

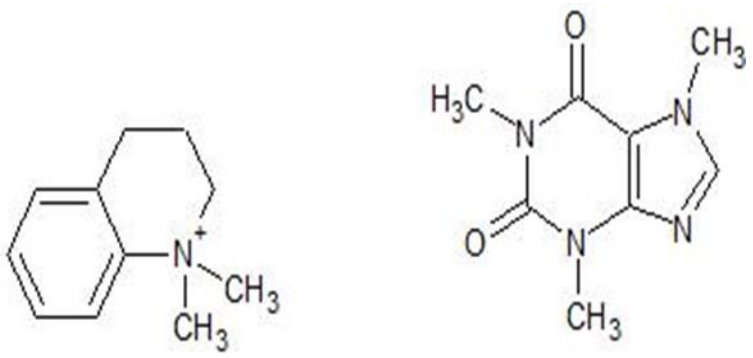

Figure 4. Structures of two alkaloid compounds, a Diene (Left) and Caffeine (Right).

According to Amirkia and Heinrich, (2014) some phytochemists divide alkaloids into three categories based on their skeletal structures:

1. True alkaloids are those compounds which are derived from an amino acid and a heterocyclic ring with nitrogen.
2. Protoalkaloids are compounds which have a nitrogen atom derived from an amino acid but the nitrogen atom is not part of the heterocycle.
3. Pseudo alkaloids are compounds whose basic carbon skeletons are not derived from amino acids but have nitrogen the heterocycle.

Alkaloids have diverse mechanisms of action, ranging from regulating blood cholesterol levels and lowering blood glucose, ameliorating the insulin resistant state to influencing the function of the pancreatic beta cell (Qiu et al., 2014). Alkaloids also have antibacterial and antimalarial activity (Amirkia & Heinrich, 2014). Berberine, an alkaloid, has been confirmed to possess anticancer activities by targeting AMP-activated

protein kinase (AMPK), which regulates tumor progression and metastasis (Ikan, 1991; Qiu et al., 2014). These significant biological activities have frequently led to alkaloids being one of the important active ingredients in Chinese traditional medicine (Qiu et al., 2014). Although alkaloids have played an important role in global pharmaceutics over the last 200 years, their use has not been limited to pharmaceutics only but has also extended to industry. Alkaloids such as N, N'-dioctadecanoyl ethanediamine, an anti-foaming agent, have been used in the polymer industry and methylamine hydrochloride in the tanning industry (Amirkia & Heinrich, 2014), demonstrating the wide applications of alkaloids.

A study by Kumari and Atri (2012) indicated low concentrations of alkaloids in Termiphilous mushrooms. A different study also screened *Oxyporus populinus* and *Psathyrella atroumbonata* for mycochemical properties and found a moderate presence of alkaloids. The presence of alkaloids in mushrooms illustrates their pharmacological importance (Kumari & Atri, 2012).

2.4.3 Polysaccharides

Mushroom compounds such as lentinan, Krestin, lectin, psilocybin, ganoderic acids, lacasses and schizophyllan are mostly protein and polysaccharide complexes (Patel & Goyal, 2012; Valverde, Hernàndez-Pérez & Paredes-López, 2015). These compounds are recognized as non-self-molecules by the organism, which stimulates the immune system to induce reactive oxygen species (ROS) (Valverde et al., 2015). Mushroom compounds also induce a host of other immunological responses such as inhibiting mitotic kinase, antimitosis, apoptosis, angiogenesis and topoisomerase inhibition. The most popular and compelling fungal medicinal of these are the polysaccharides. Polysaccharides such as β-glucans make up a big part of the fungal cell wall and are easily obtained since they are excreted into the growth medium (Valverde, Hernàndez-Pérez & Paredes-López, 2015). B-glucans are very versatile and have broad spectrum activity (Xu, Wang & Ng, 2012). B-glucans have antifungal activity that is similar to their anticancer activities and is mediated by binding to dectin-1, albeit by an unknown mechanism. The mode of action of β-glucans was unknown for some time but it has been discovered that they act through pathogen associated molecular patterns (Lee & Kim, 2014).

Of the three most popular mushrooms, *Lentinus edodes*, *Ganoderma lucidum* and *Trametes* species, more research has been done on the *Trametes* (Hobbs, 2004; Lee et al., 2012). At least 90 % of studies that have been done on *Trametes* species, especially

T. versicolor, focused on its anticancer properties. Different extracts from this mushroom have been appropriated and even patented as commercial antitumor and adjuvant cancer therapies (Patel & Goyal, 2012).

Researchers have relied heavily on polysaccharides from medicinal mushrooms to obtain immunomodulating and anticancer drugs. The development of compounds like Lentinan, Schizophyllan and Krestin has been hampered by the high molecular weight of these polysaccharide extracts weighing between 100 000 – 0.5 million Da. Since these compounds are too big to be synthesized chemically, they are still extracted from biomass such as fruit bodies and cultured mycelium or broth (Wasser, 2011). However, pharmaceutical companies prefer low weight molecular compounds which target processes such as apoptosis, angiogenesis, metastasis, cell cycle regulation and signal transduction cascades because they are easier to synthesize (Wasser, 2011).

2.4.3.1 Yunzhi

One such extract is Yunzhi, a polysaccharo-peptide (PSP) which is active against hormone responsive prostate cancer. It is even suggested that this compound has the potential to restrict prostrate tumors progressing from the hormone dependent to the hormone refractory state (Patel & Goyal, 2012). This polysaccharopeptide is a complex of protein and polysaccharide, with 56.1 % β-glucan content and 18 amino acids,

including glutamic acid and aspartic acid. Although not shown to directly kill cancer cells, this compound causes immunomodulatory effects which strengthen immunity and quality of life by reducing side effects caused by radiotherapy and chemotherapy (Yang et al., 2005; Lee et al., 2012). Interestingly, PSP was also shown to inhibit Human Immunodeficiency Virus type 1 (HIV-1) reverse transcriptase and protease enzymes (Ng & Wan, 2006; Lee et al., 2012).

2.4.3.2 Krestin

Krestin is a polysaccharide peptide extracted from *T. versicolor*. This compound inhibits the proliferation of cancer cells both in vitro and in vivo, even at concentrations as low as 20 mg/l, with an IC 50 value of 4.25 mg/l for human hepatoma cancer (QGY) cell lines (Cai et al., 2010; Patel & Goyal, 2012). Krestin is also known as polysaccharide-K or as polysaccharide-kureha (Fisher & Yang, 2002). It has been used for chemo-immunotherapy in Asia for over 3 decades and in Europe too. PSK is used in the treatment of gastric, esophageal, colorectal, breast and lung cancers (Fisher & Yang, 2002; Jiménez-Medina et al., 2008; Sun, Rosendahl, Wang, Wu & Andersson, 2012; Lee et al., 2012). PSKs may not kill cancer cells directly but they are biological response modifiers, allowing the host to respond against the tumor. Although the exact mode of action is not known, increased Natural killer (NK), lymphocyte-activated killer (LAK) cell and cytokines have been reported in vitro and in vivo after PSK treatment. Furthermore, PSK is also an antioxidant, meaning it can defend the host from oxidative

stress. With the carcinogenic nature of radiotherapy and chemotherapy, PSK can help prevent secondary malignancies (Fisher & Yang, 2002; Jiménez-Medina et al., 2008; Lee et al., 2012). Enhanced immunity, antiviral effects, improved quality of life after invasive surgery and increased survival rates have been observed, especially in colorectal and stomach cancers. With little or no side effects published, this seems like a miracle drug. However, safety of use by pregnant women, amongst others, still needs to be determined (Hobbs, 2004).

2.5 Ethnomycology

Okhuoya and Akpaja (2005) define ethnomycology as the study of the indigenous knowledge on the uses of mushrooms, as well as the legends with which they might be associated. Furthermore they state that while some of the practices cannot be explained scientifically, a number of them such as medicinal and nutritional quality can be validated with the scientific method. Indigenous people are normally well acquainted with the habitat where different mushrooms grow, as well as the periods at which they occur (Harsh, Rai & Soni, 1999) therefore, ethnomycology is an easier and more effective method compared to studying the medical relevance of all the mushrooms of the world (Okhuoya & Akpaja, 2005). Consequently, recent years have seen research focus shifting towards ethno-medicinal resources to develop novel products and drugs to treat various ailments. Natural resources also offer medicines that are safer than synthetic drugs. This said it is crucial that the traditional knowledge of communities is

recorded and published as scientific data before resources are lost with the reducing natural habitats and the passing away of older generations who possess great knowledge about these resources and their medicinal uses (Azliza, Ong, Vikineswary, Noorlidah & Haron, 2012).

Generally, there exists a difference in using natural resources to treat illness. For example, the *Parkia speciosa* root is used to treat headache in one part of Malaysia whilst it is also used to treat hypertension and diabetes in a different part of Malaysia. This dissimilarity exists despite the fact that it holds the same vernacular name in both villages (Azliza et al., 2012). Most traditional medicines are administered orally through decoction, although poultices and topical applications are also common (Azliza et al, 2012).

Kadhila-Muandingi and Chimwamurombe (2012) reported that in the Oshana and Ohangwena regions of Namibia different mushrooms are used in varying applications; *Termitomyces schimperi* and *Termitomyces sagittiformis* are mixed with oil and applied to treat wounds in children's heads, *Amanita* species are used on arrow heads for hunting. During the same study it was also discovered that in the Oshana and Ohangwena regions of Namibia men had greater knowledge about the medical applications of mushrooms than the women did, with the elderly having more reliable experience and more people using mushrooms purely for food and not as medicine. The

information obtained from traditional or indigenous knowledge can be followed up with studies to determine the chemical components in the natural resources. Efficacy in treating disease can also be tested (Azliza et al., 2012).

2.6 Identification of fungi using the Internal Transcribed Spacer region

Molecular identification and characterization of wood decaying fungi is often carried out using the 18S ribosomal RNA and internal transcribed spacer region RNA to determine genetic relationship and diversity (Rajala, Peltonieni, Hantula, Mäkipää & Pennanen, 2011). Being one of the most frequently used genes in phylogenetics, the small subunit (SSU) 18S rRNA is an important marker for random target PCR. This gene is widely used because it is easily accessible due to its highly conserved flanking regions, which allow the use of universal primers. Even in the smallest organisms, excessive amounts of DNA template for use in PCR are available (Meyer, Todt, Mikkelson & Lieb, 2010). This is as a result of the 18S gene being repetitively arranged within the genome. The 18S gene is part of the ribosomal functional core and goes through the same selective conditions in all beings. The ITS is the piece of non-functional RNA found between the structural ribosomal rRNA on a common precursor transcript (Figure 5). The ITS region, now probably the most widely sequenced fungal DNA region is most useful for molecular systematics at both species level and within species. It has a higher degree of variation compared to other gene regions of rDNA and has proven to be useful in

explaining relationships among species and closely related genera (Nilsson, Kristiansson, Ryberg, Hallenberg & Larsson, 2008).

There is difficulty in identifying fungi by morphologic characteristics alone. Molecular techniques are of great help in such cases. In most Basidiomycetes and many Ascomycetes, ribosomal DNA is made up of repeat units composed of major transcripts (18S, 5.8S and 28S) and an internal transcribed spacer (ITS) region (Nilsson et al., 2008). The ITS region has a higher degree of variation which can be useful as markers for the discrimination of fungal species and has been widely used in molecular systematics to differentiate at species levels or sometimes even within species (Kim, Kim, Lee, Park & Ro, 2013). The ITS region can be used to successfully develop species-specific primers, as Kim et al. (2013) have done. They had fungi of unknown identity in their sample. In order to identify it they amplified the whole ITS1-5.8 S rDNA regions of all the different fungi and sequenced the amplified DNA to get the different ITS sequences of the unknown fungi. The sequences were used in BLAST to reveal the identity of the unknown fungi. The sequences were further analyzed by multiple sequence alignment and specific primers for each fungal strain were developed from the sequences obtained. The high variability of the ITS region allowed species-specific fungal primer sets to be designed.

Schoch et al. (2012) compared three subunits from the nuclear ribosomal RNA cistron with three representative protein-coding genes. They found that the protein-coding gene regions showed a higher percentage of correct identification compared to ribosomal markers, which showed low PCR amplification. Schoch et al. (2012) further found that from the ribosomal cistron, the ITS region was more likely to successfully identify the broadest range of fungi, having the most clearly defined barcode gap between inter- and intraspecific variation. Despite having superior species resolution in some taxonomic groups like Ascomycete yeasts, the nuclear ribosomal large subunit was considered inferior to the ITS. This is mainly because of the poor species level resolution of nuclear ribosomal large subunit in fungi. The ITS region was formally proposed as the primary fungal barcode marker to the Fungal Barcoding Consortium in 2012 (Schoch et al., 2012; Bellemain et al., 2010).

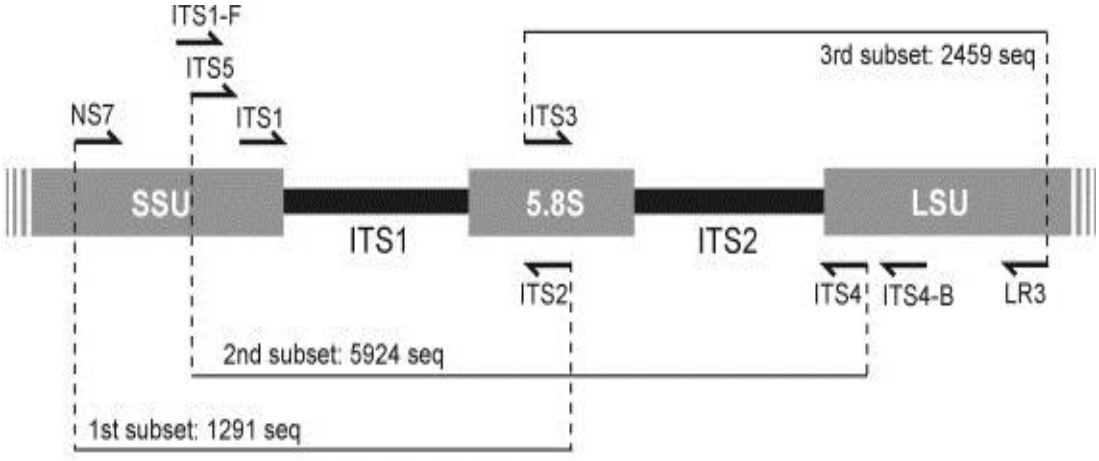

Figure 5. ITS region of Fungi (Bellemain et al., 2010).

Traditional mushroom taxonomy was mainly based on morphological description of the fruit body, host specificity and geographical distribution. However mushrooms in the *Trametes* genus have a similar morphology and have proved a challenge to identify them based on this traditional technique. As a result the *Trametes* genus is considered one of the most confused in the Polyporaceae. Mycologists have since turned to molecular techniques to explain the taxonomic challenges in *Trametes* and related genera (Oyetayo, 2014).

A study by Oyetayo (2014) to analyze the ITS region of *Trametes* species from Nigeria revealed genetic differences in the Nigerian species and those from GenBank. The Nigerian *Trametes* species were divided into three clades meaning that they were not as closely related to each other. In another study that compare phylogeny of European and American *Trametes* species, all except *T. cervina* were placed in one clade (Tomšovský, Kolařík, Pažoutova & Homolka, 2006). These differences might occur because of geographical and environmental factors. Geographic factors are responsible for fungal diversity at a regional level in a radius of 1000-4000 km while environmental factors may cause diversity at a local level within a radius of less than 1000 km (Oyetayo, 2014).

It is evident that mycologists are divided in their taxonomic classification of the *Trametes* genus. Justo and Hibbet (2011) recommend that a broad generic concept for

Trametes is the preferred taxonomic and nomenclatural option. Welti et al., (2012) are of a different opinion. They argue that taxonomic arrangement should consist of four genera within *Trametes* based on the monophyly of groups inferred from ITS and RPB2 sequences and four differences in morphology. These differences include a lineage corresponding to 'genuine' *Trametes* species, *Pycnoporus* species, *Artolenzites* Falck., "Lenzites" elegans (Spreng.) Pat., and *Leiotrametes* Welti and Coartec, including *Trametes menziesii* (Bark.) Ryv., *T. lactinea* (Berk.) Sacc. and *Leiotrametes* species.

Species phylogeny based on nrITS data supports that *Trametes versicolor, T. pubescens, T. ochracea* and *T. ectypa* are from a clade with high morphological similarity. On the contrary, Tomšovsky and Homolka (2004) discovered that *T. versicolor, T. ochracea* and *T. pubescens* were incompatible sexually. Some argue that species delimitation occurs due to differences in the color and texture of the pileus (Gilbertson & Ryvarden, 1987 as in Carlson, Justo, & Hibbet, 2014). The above clearly illustrates the need for proper studies on ecology, identification and medicinal use of indigenous mushrooms (Oyetayo, 2011).

CHAPTER 3: Materials and Methods

3.1 Research Design

The experimental design used in this study consisted of three stages which had a phylogenetic and chemical approach (Figure 6). The first stage was to collect indigenous *Trametes* species from their natural hosts in Northern Namibia, extract genomic DNA for sequencing and to construct a phylogenetic tree from the unpublished indigenous *Trametes* sequences together with sequences from GenBank. The second stage was to perform mycochemical and antibacterial screens on the indigenous *Trametes* extracts. The final stage involved acquiring ethnomycology knowledge from the local people in Northern Namibia by the use of questionnaires.

This study used qualitative and quantitative methods to obtain data. Qualitative data was obtained from questionnaires, mycochemical screens and fungal ITS sequences. Quantitative data was obtained from the antimicrobial screens.

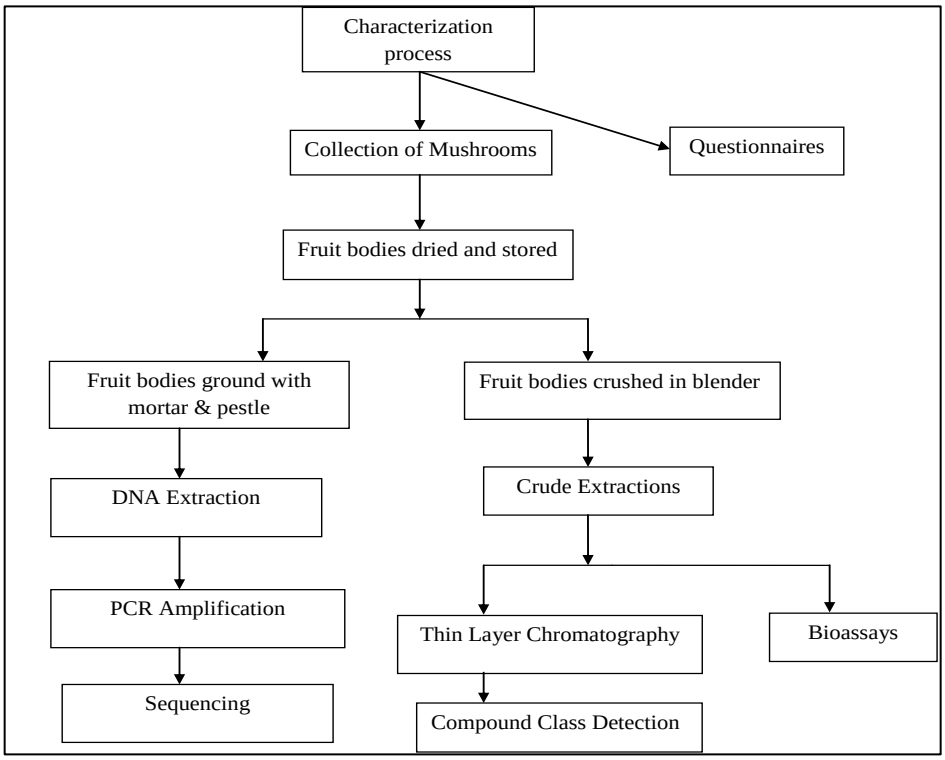

Figure 6. Characterization processes of Namibian indigenous *Trametes* species.

3.2 Study Site

The study area (Figure 7) was chosen because the Northern regions of Namibia receive significantly more precipitation than the rest of the country and there is more vegetation of trees (Rohde & Hoffman, 2012). This provides a habitat for *Trametes* species which grow on dead branches, usually of broad-leaved trees in relatively dry tropical areas.

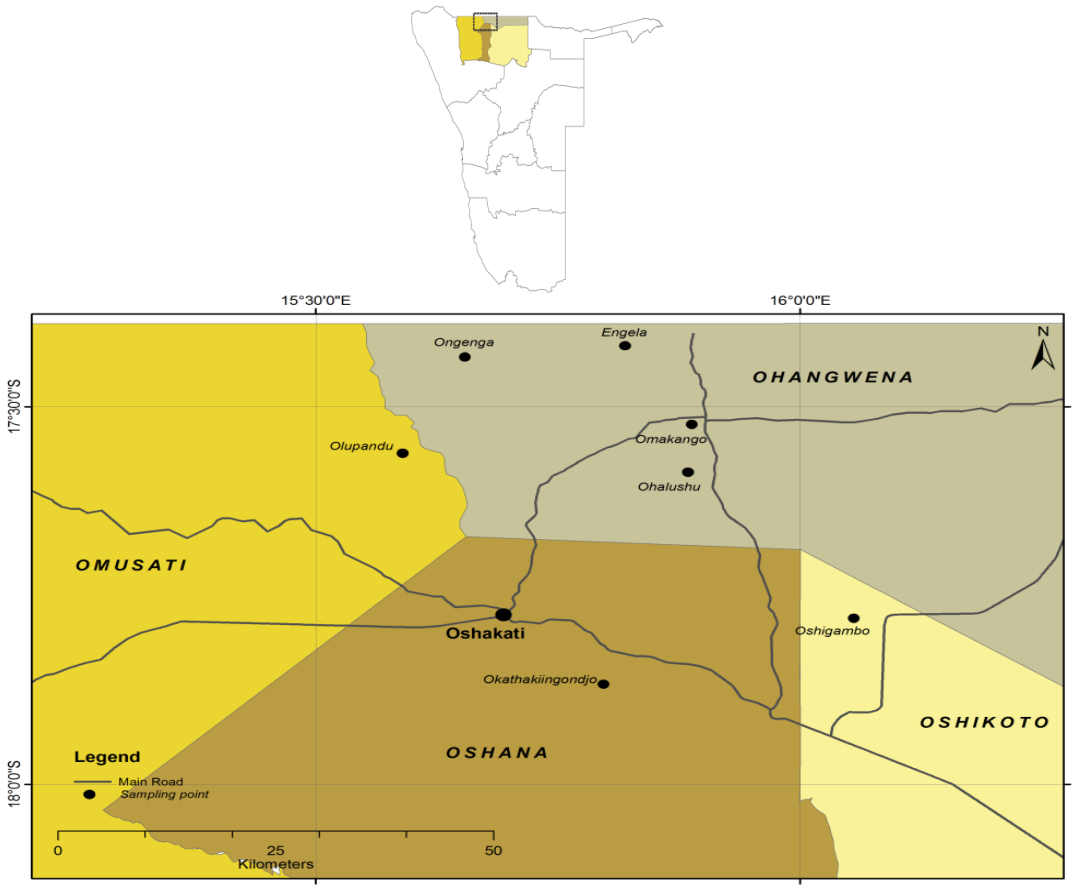

Figure 7. Study sites in Northern Namibia where indigenous *Trametes* species were collected.

3.3 Sample

The sampling method used in this study was non-probability convenience sampling. This method was chosen in order to reduce travel costs and time because of the vastness of the study area. Sampling was done by driving around the villages and stopping at areas with high population of wood stumps and walking along fences to look for the

mushrooms. Once *Trametes* mushrooms were spotted, the fruiting bodies were carefully pulled off the substrate by holding the mushroom and pulling gently. The mushrooms were put in brown paper bags and the name of region and village, as well as substrate type (wood stump, pole, etc.) was recorded. Pictures were also taken of the *Trametes* species in their natural habitat. Caution was taken to not harvesting all the mushrooms from the same substrate to ensure continued spore dispersal.

3.4 Procedure

3.4.1 Samples Collection and Identification

A collections permit was obtained from the Ministry of Environment and Tourism in Namibia. The indigenous mushrooms were collected from the natural habitat of Northern Namibia during late March and early April 2014. Dry *Trametes* species were collected from wood stumps and fence poles around the villages and along the roadside in three regions, namely Ohangwena, Omusati, and Oshana region and a small section of Oshikoto region. The harvested fruiting bodies were identified as *Trametes* species according to Van der Westhuizen and Eicker (1994) and Læssøe (2013). The samples were recorded and kept in khaki paper bags labeled as *Trametes*, name of village and region, as well as host. In order to create a simple coding system, the villages were assigned letters of the alphabet and each fruiting body a number. Table 1 shows a summary of the information collected during sample collection.

Table 1. The locations where fruiting bodies were harvested.

Region	Village	Host	Code
Ohangwena	Ohalushu	Wood in the field	$C_{(n=4)}$
Oshana	Okatha	Fence poles	$B_{(n=4)}$
Omusati	Okalumbi	Wood in the field	$E_{(n=5)}$
Ohangwena	Ohalushu	Dead tree stump	$D_{(n=11)}$
Ohangwena	Ohalushu	Dead tree stump	$I_{(n=4)}$
Ohangwena	Ongenga	Fence poles	$L_{(n=5)}$
Oshikoto	Oshigambo	Wood along fence	$A_{(n=3)}$
Ohangwena	Ohalushu	Wood in the field	$J_{(n=9)}$
Ohangwena	Ohalushu	Dead tree stump	$K_{(n=6)}$
Omusati	Olupandu	Fence poles	$H_{(n=6)}$
Ohangwena	Omakango	Wood in the field	$M_{(n=9)}$

n = number of fruiting bodies harvested per site

3.4.2 Samples Preparation and Extraction

The collected mushrooms were dried in a cool shade outside for 6 hours and stored in a cool dry place (room temperature: 20-25 °C). All visible sand and wood particles were removed with a new tooth brush before grinding the mushrooms in a commercial blender (Platinum Stand Blender 1.5 l). The mushroom powder was stored in a sterile glass bottle at room temperature until further use.

Organic and aqueous extractions of the *Trametes* mushrooms were prepared with methods adopted from Molefe-Khamanga et al. (2012). Hot water extract (HWE) was performed with 80 °C distilled water while cold extraction with organic solvent was done by using 10 g of the powdered material and 200 ml of the solvent in ascending order of polarity. The mushrooms were extracted sequentially with hexane, dichloromethane (DCM), ethyl acetate and methanol to yield extracts of different polarity. The solvent mixtures were stirred on a magnetic stirrer overnight and filtered with Whatman no 1 filter paper. The filtrates were dried under reduced pressure using a rotary evaporator at 40 °C and 150 rotations per minute (rpm) for the organic extractions and 60 °C and 85 rpm for the HWE before freeze drying. The remaining traces of moisture were dried in a fume hood and the extracts were weighed and stored in airtight vials at 4 °C until further use.

3.4.3 Thin Layer Chromatography

TLC methods were adopted from Harbone (1998). The extracts were analysed on small (55 mm x 105 mm) scale pre-coated silica gel plates (Merck TLC Silica gel 60 F_{254}). Chromatograms were developed in ethyl acetate: toluene (2:1; 1:2; 3:1; 1:3; 1:1), hexane: methanol (1:1; 2:1; 1:2; 2:3; 1:3; 3:1; 4:3), ethyl acetate: hexane (4:1; 1:1; 2:1; 1:2; 1:3; 3:1; 1:4; 2:3; 3:4; 2:5), methanol: DCM (1:1; 1:2), ethyl acetate: toluene: methanol (8:4:1; 3:2:1), ethyl acetate: methanol (1:1), and hexane: ethanol (2:1; 1:2). Ethyl acetate: toluene in a ratio (v/v) of 1:2 was found to be the best solvent system for

the non-polar compounds separated in this study. Labeled 250 ml beakers containing the different developing systems were covered with a watch glass for a few minutes, while preparing the TLC plates, to saturate the air in the beaker with vapor of the solvent. The TLC plates were placed in the beaker (without bringing the spots in contact with solvent) and watch glass replaced. The compounds moved up the TLC plates at different rates according to their polarity. When the solvent had reached the top of the plate it was removed and the solvent front marked immediately before air drying. The separated compounds were invisible to the naked eye, thus viewing was done under ultra violet (UV) light at 254 nm and 366 nm wavelengths. The observed patterns were marked with a pencil.

3.4.4 TLC Visualization Reagents

3.4.4.1 Iodine Vapor

Iodine vapor was prepared by placing six iodine crystals in a beaker containing ethyl acetate: toluene in a ratio (v/v) of 1:2. The beaker was covered with a watch glass to saturate it with vapor from the iodine crystals. Lastly, TLC plates were developed by placing them in the beaker which was saturated with vapor (Susmitha, Vidyamol, Ranganayaki, & Vijayaragavan, 2013).

3.4.4.2 Test for Flavonoids

The chromatograms were first developed in the mobile phase of ethyl acetate: toluene in a ratio (v/v) of 1:2 and dried. Each plate was then sprayed with 10ml of freshly prepared 1 % (w/v) 99 % ethanolic $AlCl_3$ before drying. Finally, viewing was done under UV light at 365 nm to observe for any change in colour (Susmitha et al., 2013).

3.4.4.3 Test for Alkaloids

After developing new chromatograms and drying them, they were each sprayed with 10 ml of freshly prepared Wagner's reagent and dried. For viewing, UV light was used at 365 nm and 254 nm. Any observations and changes in colour were recorded (Ikan, 1991).

3.4.4.4 Test for Coumarins

Methanolic potassium hydroxide 10% was prepared by dissolving 2 g potassium hydroxide (KOH) in 20 ml methanol. The newly prepared chromatograms were sprayed

with the 10 % (w/v) methanolic KOH and dried. Lastly, viewing was done under UV light at 365 nm and 254 nm (Nafuka & Mumbengegwi, 2013).

3.4.4.5 Test for Saponins

The chromatograms were first developed in the mobile phase of ethyl acetate: toluene in a ratio (v/v) of 1:2. After drying, they were dabbed with vanillin solution and lightly heated over a hot plate. Any observations such as colour change were recorded.

3.4.4.6 Anisaldehyde

The chromatograms were first developed in the mobile phase of ethyl acetate: toluene in a ratio (v/v) of 1:2. After drying, they were dabbed with Anisaldehyde solution and lightly heated over a hot plate. Any observations such as colour change were recorded (Pyka, 2014).

3.5 Antimicrobial tests

3.5.1 Microorganisms and Growth Conditions

The disc diffusion method was used to test the antimicrobial activity of the extracts on *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 11774), *Staphylococcus aureus* (ATCC 25923), *Alcaligenes faecalis* (ATCC 8750) and *Neisseria meningitidis* (ATCC 35561). Nutrient broth was inoculated with lyophilized bacterial cells of test microorganisms. The cultures were incubated at 30 °C for 24 hours and transferred to new nutrient broth. The final inoculum was prepared by pipetting 2 ml of 90 % saline solution to new tubes and inoculating with bacterial cultures using a loop. Nutrient agar was prepared according to manufacturer's instructions and poured into 90 mm petri dishes. The nutrient agar plates were inoculated with 100 µl of the prepared saline bacterial suspension. Sterile glass rods were used to spread the inoculum evenly onto the agar.

3.5.2 Disc Diffusion Method

DCM and methanol extracts were dissolved in DMSO to make a 25 mg/ml concentration and HWE was dissolved in distilled water to make a 25 mg/ml concentration. Plates of Nutrient agar plates were prepared according to manufacturer's instructions. The nutrient agar plates were inoculated with 100 µl of the prepared saline bacterial

suspension. Sterile glass rods were used to spread the inoculum evenly onto the agar. Sterile 6 mm filter paper discs were impregnated with 10 µl of the extract and placed onto the inoculated agar. The tests were done in triplicate by placing three discs impregnated with the same extract in one plate with a fourth one impregnated with the negative control. DMSO and distilled water were used as negative control and oxytetracycline was the positive control. The plates were incubated at 4 °C for 2 hours to allow the extract to diffuse into the agar before they were incubated at 37 °C. Readings were taken after 18 hours of incubation and zones of inhibition measured with a millimeter ruler and recorded.

3.6 Ethnomycology of *Trametes* Mushroom

A questionnaire was used to obtain indigenous knowledge of local people in Northern Namibia on the medicinal uses of indigenous *Trametes* mushrooms. The questionnaire was administered in face to face interviews in the Oshiwambo language. The villages and households were selected according to accessibility and in each household only people above 30 years old were interviewed. Some individuals chose only to answer the section on *Trametes* mushrooms because they were busy. The questionnaire was semi-structured and allowed for dialogue with the respondents. Respondents were also shown dry fruit bodies of *Trametes* for easier identification. See Appendix 2 for a copy of the questionnaire.

3.7 Fungal DNA Extraction

Trametes samples were ground to powder using sterilized mortar and pestles. A Qiagen DNeasy® Plant Mini Kit was used for DNA extraction. Fungal genomic DNA was extracted according to manufacturer's protocol, with minor adjustments.

A weight of 100 mg of sample was mixed with 400 µl Buffer AP1 and 4µl of Rnase A by vortexing. The mixture was incubated at 65 °C for 10 minutes with inversion of the tubes 2 to 3 times during incubation to allow sufficient lysis. After incubation 130 µl Buffer P3 was added to the lysate and after thorough mixing it was incubated on ice for 5 minutes. This step was followed by centrifuging for 5 minutes at 14 000 rpm. The lysate was pipetted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at 14 000 rpm. The flow-through was transferred to a new tube, taking care not to disturb the pellet formed, and 300 µl of Buffer AW1 was added and mixed by pipetting. Transferring 650 µl of the mixture into a Dneasy Mini spin column placed in a 2 ml collection tube, the mixture was centrifuged at 8000 rpm for 1 minute. The flow-through was discarded and the spin column was centrifuged again for 1 minute at 8000 rpm. The spin column was placed into a new 2 ml collection tube and 500 µl Buffer AW2 was added before centrifuging for 1 minute at 8000 rpm. The flow-through was discarded and 500 µl Buffer AW2 was added once more. This was followed by centrifuging for 2 minutes at 14 000 rpm. Carefully, without bringing the column in

contact with the flow-through, the spin column was transferred to a new 1.5 ml Eppendorf tube and 50 µl of Buffer AE solution was added. The spin column was incubated at room temperature for 5 minutes and centrifuged for 1 minute at 8000 rpm. Another 50 µl Buffer AE was added for final elution. The last step was to incubate the spin column again at room temperature for 5 minutes and centrifuging at 8000 rpm for 1 minute. The flow-through is the extracted genomic DNA.

PCR amplification was performed with a 25 µl volume consisting of 12.5 µl DreamTaq Green PCR Master Mix (2X), 10.5 µl nuclease free water, 1 µl ITS1-F primer (CTTGGTCATTTAGAGGAAGTA), 1 µl ITS2G primer (GCTGCGTTCTTCATCGATGC) and 1 µl DNA. PCR conditions were as follows: Pre-denaturation at 95 °C for 4 mins, denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 2 mins for 35 cycles. This was followed by a final extension of 72 °C for 7 mins.

Gel electrophoresis was prepared after genomic DNA extraction and PCR. Gel electrophoresis was performed in 0.5 % Tris-borate EDTA (TBE) buffer. The DNA gel was prepared by dissolving 1 g agarose gel in 100 ml TBE buffer (1 %) and completely dissolving it by heating in a microwave for 1 minute. After slight cooling 2.5 µl ethidium bromide was added to the gel before casting in a tray. Gel electrophoresis was run at 110 V for 60 minutes after which the gel was viewed under UV light.

3.7.1 Sequencing

Unpurified PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd for sequencing using ITS1 and ITS2 primers. Inqaba Biotechnical Industries (Pty) Ltd performed the DNA template purification and isolation as well as the sequencing reaction. Once the sequences were received back from South Africa, cleaning and alignment of the sequences was done in preparation for phylogenetic analysis.

3.8 Data Analysis

WinRAR archiver Software was used to open the ABI files containing nucleotide sequences after which sequence cleaning and editing was done in Chromas Lite201. A Basic Local Alignment Search Tool (BLAST) was done in NCBI database to identify the nucleotides (Altschul, Gish, Miller, Myers & Lipman, 1990). A Local Alignment of the ITS1 and ITS2 fragment sequences was performed in Bioedit to create contig sequences. A BLAST search was performed on the 44 contig sequences using the Unite Analysis for Fungal species database and NCBI GenBank database. The unpublished sequences as well as the sequences obtained from GenBank were aligned with Bioedit and Clustal W. For phylogenetic analysis, the Maximum Likelihood (ML) analysis was

performed in MEGA version 6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). The phylogenetic tree was reconstructed using the Neighbour Joining method and reliability for internal branch assessment was done using the ML bootstrapping method with 500 ML bootstrap replicates.

The SPSS Software was used to analyse the information obtained from questionnaires. Analysis of Variance (ANOVA) was used to test for significant difference in ethnomycology of *Trametes* mushrooms in Oshana, Ohangwena and Omusati regions (Appendix 6).

The antimicrobial activity of *Trametes* extracts was assessed in terms of the inhibition zone diameter of the bacteria. The antimicrobial activity of indigenous *Trametes* species was then expressed as mean ± standard deviation (SD) in mm, where n = 3.

3.9 Research ethics

Adherence to University of Namibia research ethics was observed at all times. Research ethics clearance was obtained from the UNAM School of Postgraduate Studies and a specimen collection permit from the Ministry of Environment. Permission was sought before entering ‘mahangu’ fields to look for *Trametes* mushrooms. During interviews the Headman of the village (or his representative) was also approached and informed

about the intentions of the study. It was clarified to the respondents who took part in the interview what the study was about and their permission obtained before commencing with the interviews. The local people were also interested in the results of the study and indicated that they would like to get feedback from the interviews. The student therefore explained that the results of the study will be made available in the form of pamphlets and educational visits back to the communities. Laboratory safety measures and safe disposal of used chemicals and reagents was adhered to.

CHAPTER 4: Results

4.1 Samples Collection and Identification

All mushrooms collected in the regions were found on wood, mostly wood stumps and poles used in fencing, and identified as *Trametes* species based on their morphological features. Since the mushrooms were found growing on wood (Figure 8), it was difficult to identify specific tree host species, although one can look at the surrounding vegetation and assume that the wood might possibly come from the same trees. Total sites sampled were 13 but samples from 2 sites were discarded due to insufficient data (Table 1). Oshana region recorded the lowest number of fruit bodies collected from 3 sites while Ohangwena region had the highest number of fruiting bodies collected from 11 sites (Figure 9). About 70 fruit bodies were collected (Table 1).

Figure 8. *Trametes* mushrooms growing on wood.

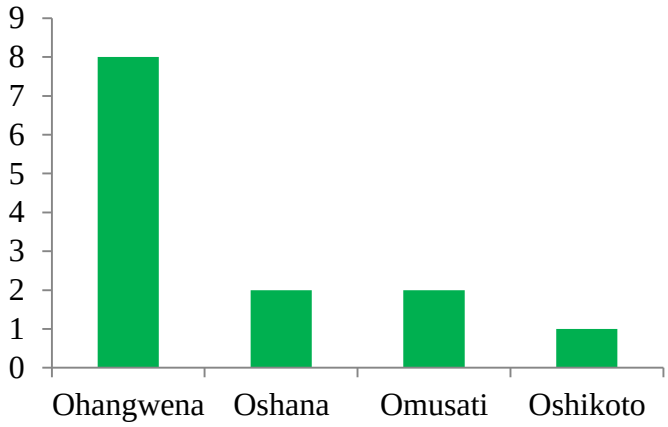

Figure 9. The number of sites where *Trametes* species were harvested per region.

The mushrooms collected presented a morphology characteristic of *Trametes* species although a high variation was observed in size and colour of fruit bodies, size of pores, concentric zones and rigidity of mushroom upon breaking or tearing. Based on these morphological differences, at least four *Trametes* species were identified, as they are displayed in figure 10. Specimen A was always grey basidiocarp with 73 mm diameter in colour with 1 to 2 mm wide pores underneath. Specimen B was a thin and dark brown basidiocarp 70 mm in diameter with black prickly 'hairs' on the top surface and 1mm sized pores on the hymenium. Specimen C was the smallest with 25 mm diameter basidiocarp of the four types observed with 1 mm sized pores. The basidiocarp was covered with distinct zonate areas with different shades of grey, white and black. Specimen D basidiocarp was 45 to 75 mm in diameter with zonate areas of shades of tan, grey, black and white colour and 2-3/mm. The hymenium was covered with many

small white pores ranging from 2-3 pores/millimeter. The mushrooms lacked a distinct pileus as they were attached directly to their host.

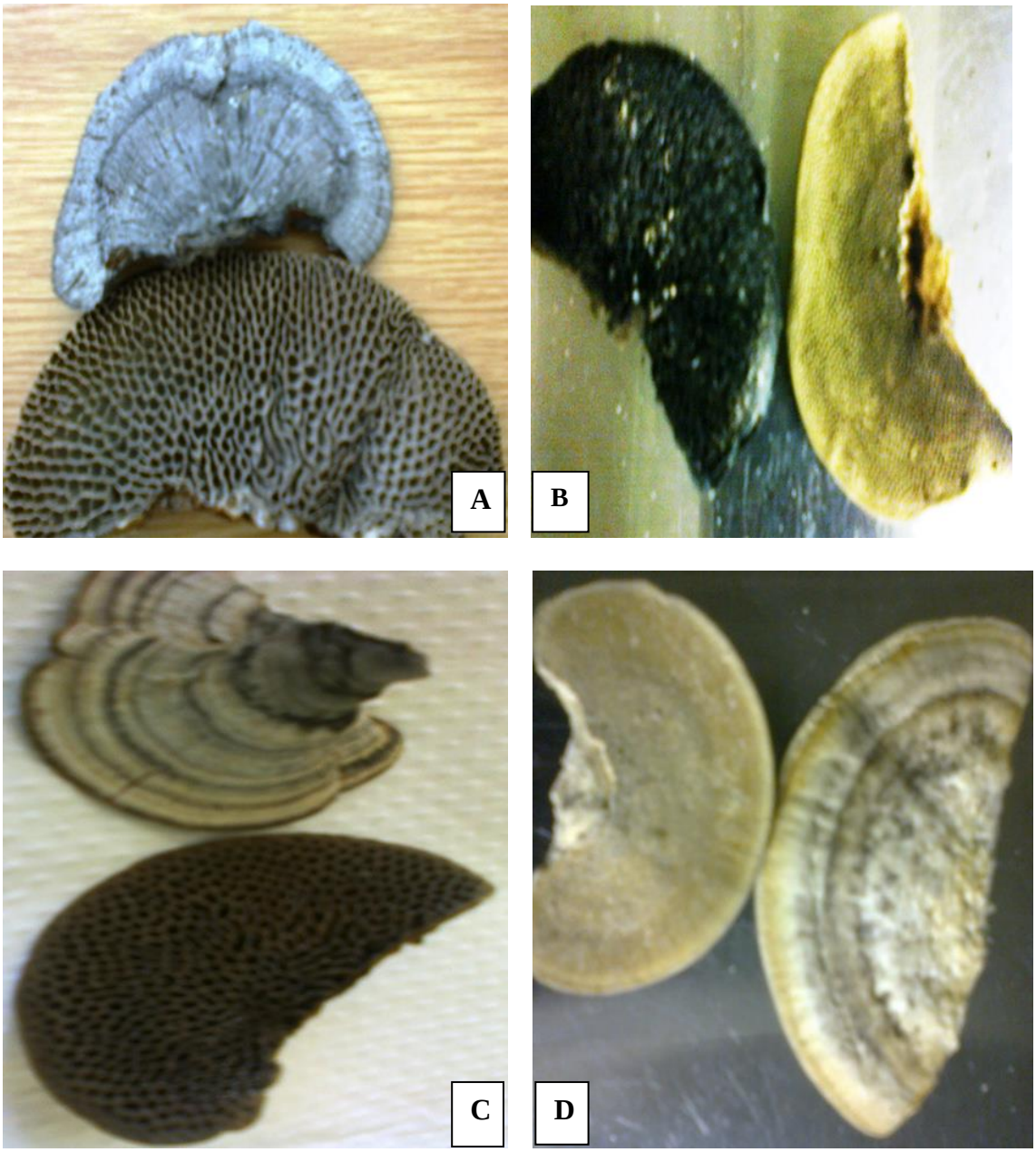

Figure 10. The mushrooms collected showed four distinct morphologies. **A:** grey basidiocarp with hexagonal pores. **B:** fine black hair like structures with fine regular

pores. **C:** distinct zones, white to grey colour and large regular pores. **D:** distinct zones with tan, grey, white or black colour and fine pores.

4.1.1 Morphological features of the mushrooms

The images in Figure 10 are not to scale and therefore do not show a clear representation of size. However, the images clearly show the variation in colour and pore size and shape. Interesting to note is the characteristic 'bracket' shape common to the polypores.

Basidiocarp: The fruit bodies showed variety in colour, size and concentric zones with a uniform bracket shape. The basidiocarp diameter ranged between 2.5 cm to 8 cm with colours ranging from brown or tan to grey and even some fruit bodies that had short and prickly 'hairs' on the upper surface. Distinct concentric zones were visible in many of the basidiocarps, with different shades of colour for each zone (Figure 10 C, 10 D).

Pores: There was also variety observed in the size of pores for the different mushrooms collected. Some mushrooms had a yellow/tan hymenium layer with small fine pores. The mushrooms with a darker hymenium exhibited bigger pores ranging from 2-3 pores/mm to pores slightly bigger than 1mm in diameter (Figure 10 A, 10 D).

Pileus: The mushrooms lacked a distinct pileus as they were attached directly to their host. This type of mushroom which lacks a pileus is called sessile (Figure 10).

4.2 Samples Extraction

After sequential extraction was performed the products were weighed and yielded different extract quantities (Table 2). The hexane extract yielded a yellow crystalline substance, ethyl acetate and DCM yielded a dark brown fine powder while methanol produced a dark and slightly oily extract.

Table 2. Description and yield of organic and aqueous extracts from 10 g of dried *Trametes* material.

Solvent	Extract Appearance	Yield (mg/g)
Hexane	light yellow crystalline	0.005
Ethyl acetate	light brown fine powder	0.001
DCM	dark brown fine powder	0.090
Methanol	oily dark brown-reddish	0.072
Hot Water	dark brown-reddish	0.060

4.3 TLC Visualization Reagents

The presence or absence of secondary metabolites in indigenous *Trametes* species from Northern Namibia was tested. Chromatograms were developed in a number of mobile phases at various ratios as given in section 3.5.3. The resulting chromatogram showed nine compounds separated. These chromatograms were treated with aluminium chloride solution and showed a purple colour at 254 nm in UV light (Figure 11). This indicates the presence of flavonoids in the extract. The Wagner's reagent test also showed positive results by producing brown spots on the TLC plate (Figure 12), indicating the presence of alkaloids. When the hexane extract was sprayed with vanillin, green and dark spots appeared after heating, indicating a positive test for triterpene saponins (Appendix 5). The anisaldehyde test was also positive. There were no coumarins detected in the extracts (Table 3).

Table 3. Presence/absence of coumarins, alkaloids and flavonoids in indigenous Namibian *Trametes* species.

Visualization Reagent	MeOH Extract	Positive Test	Compound Tested
Iodine Vapour	Yellow with brown spots	Yellow brown zones	Universal stain
Aluminium chloride	Purple at 254 nm	Green and purple zones	Flavonoids
Wagner's reagent	Brown spots at 254 nm	Orange brown	Alkaloids
Methanolic KOH	Purple at 365 nm	Yellow zones	Coumarins
Vanillin	Green and dark spots	blue-violet, yellow	Saponins
Anisaldehyde	Green, purple spots	green, red, blue	Universal stain

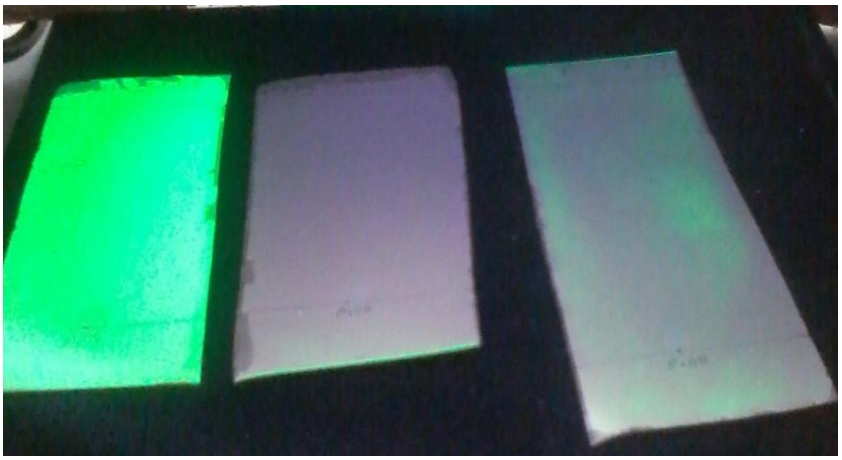

Figure 11. TLC chromatograms of the methanol extract of *Trametes* species sprayed with 1 % $AlCl_3$ ethanolic solution and viewed under UV light at 254 nm. Purple zones indicate the presence of flavonoids.

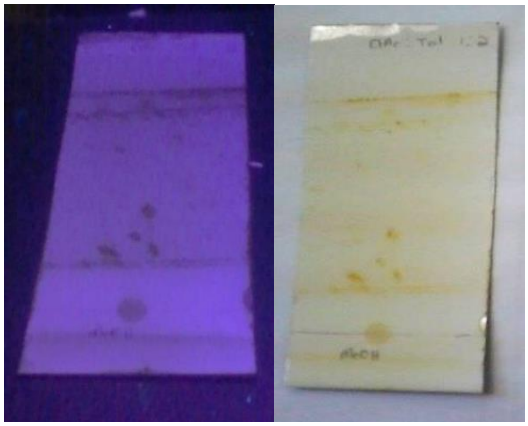

Figure 12. TLC chromatograms of the methanol extract of *Trametes* species developed in Wagner's reagent, viewed under UV light (Left) and visible light (Right).

For the antimicrobial assays, the highest zone of inhibition observed was 12 ± 1 mm by the DCM extract on *N. meningitidis.* The lowest observed was 9.7 ± 2.08 mm also caused by the DCM extract on *B.subtilis* followed by *A. faecalis* with 10 ± 0.0 mm inhibition (Table 4). The DCM extract reduced growth but did not completely inhibit *E. coli*, this is observed by the feint zones of inhibition (Figure 13, A). Interestingly no zones of inhibition were observed for the methanol and hot water extracts.

Table 4. Antibacterial activity of crude extracts from indigenous Namibian *Trametes* species.

Extract	Zones of inhibitition (mm)				
	S. aureus	***E. coli***	***B. subtilis***	***N. meningitidis***	***A. faecalis***
DCM	0.0 ± 0.0	11.3 ± 0.58	9.7 ± 2.08	12 ± 1	10 ± 0.0
Hexane	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MeOH	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
HWE	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Positive Control	33.3 ± 2.89	25.7 ± 3.79	26 ± 1.73	27 ± 2.52	27.7 ± 3.79

Values are mean ± standard deviation (SD), where n=3

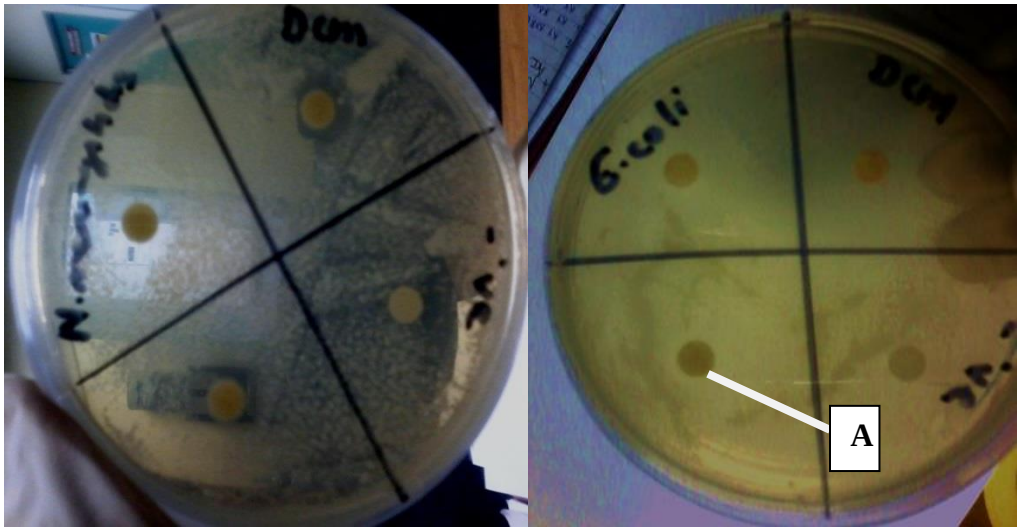

Figure 13. Antimicrobial activity of *Trametes* DCM extracts against *N. meningitidis* (Left) and *E. coli* (Right). Incomplete inhibition of *E. coli* is visible at A.

4.4 Fungal DNA Extraction

Out of 100 mushroom samples, 78 yielded good quality DNA which was amplified with the PCR. PCR products of the expected size of 500-600 bp were produced successfully for 66 samples which were then sent to Inqaba Biotec Industries for sequencing and 88 pairs of sequences of 200-250 base pairs were produced. The ITS region of nuclear rDNA from 40 *Trametes* species from Northern Namibia was used for molecular identification. The sequences obtained were aligned with sequences from GenBank. Alignment with GenBank sequences revealed a variety of identities such as C1-C4, C21 = *Coriolopsis caperata* with 99 % identity, D1-D7, D9, D11, D13 = *Trametes polyzona* with 99 % identity, E1, E3-E4 = *Truncospora macrospora* with 93 % identity, specimen F1, I2-I4, K3-K6, M6 = *Trametes spp.* with up to 99 % while E2, G6, J2, J6-J7 resemble *Trametes spp.* with 93 % identity (Table 5).

The phylogenetic tree generated from the unpublished sequences and sequences from GenBank showed some variation. The phylogenetic tree showed 8 major clades which were all supported with bootstrap values higher than 50 % (Appendix 4). Clade 1 contains Specimen D1-D9, D11, D13 and Specimen F1, I2-I4 and K3-K6 as well as GenBank sequences *Trametes polyzona* (JN164979.1), *Coriolopsis polyzona* (FJ627248.1), *T. gibbosa* (FJ481048.1), *T. villosa* (KF573031.1), *T. hirsuta* (GU062274.1), *T. maxima* (JN164918.1), *T. cinnabarina* (AB735965.1), *Pycnoporus*

sanguineus (AJ537499.1), *T. cubensis* (KJ654513.1), *T. orientalis* (AB735966.1), *T. elegans* (EU661879.1), *T. ljubarskii* (GU731579.1) and *T. marianna* (KC848334.1). Clade 2 contains Specimens E1; E3-E4 alongside *Truncospora macrospora* (JX941573.1), Clade 3 contains *Trametes tenuis* (KC414233.1), *Daedaleopsis sp.* (KF541330.1) and *Fomes fomentarius* (EF155494.1). Clade 4 contains Specimen G6 and G9 while clade 5 contains Specimen C1-C4, C21, M6-M7 and GenBank sequences *Coriolopsis caperata* (AB158316.1), *C. trogii* (KJ093492.1), *Funalia trogii* (EU273516.1), *C. gallica* (JN165013.1), *Trametes suaveolens* (FJ478094.1), and *T. trogii* (HM989941.1). Clade 6 has *Trametes hirta* (KC867359.1), *C. aspera* (KP013018.1) and *H. apiaria* (KC867362.1). Clade 7 has Specimen E2, E5 and J1, J3-J9 while Clade 8 only contains Specimen J2.

All the samples which were grouped in the Trametoid clade were supported with a high bootstrap value of 96 % (Figure 14). It was interesting to observe that these mushrooms all had tan to brown basidiocarps with distinct zones and fine pores under the basidiocarp (Figure 10 D). The remaining samples collected from Northern Namibia were distributed in 6 clades mainly alongside *Hexagonia*, *Truncospora* and more distantly *Coriolopsis spp* (Appendix 4). These samples which were not grouped in the Trametoid clade had morphologies ranging from a grey basidiocarp with hexagonal pores to fine black hair-like structures with fine regular pores and distinct zones, white to grey colour and large regular pores (Figure 10 A-10 C).

Table 5. Genomic identification of indigenous *Trametes* species from Northern Namibia based on ITS region.

Sample	NCBI	Identity	Accession Number
C1	*Coriolopsis caperata*	*99%*	KF564288.1
C2	*Coriolopsis caperata*	*99%*	KF564288.1
C3	*Coriolopsis caperata*	*99%*	HQ323692.1
C4	*Coriolopsis caperata*	*98%*	GQ372861.1
C21	*Coriolopsis caperata*	*99%*	HQ323692.1
D1	*Trametes polyzona*	*99%*	JN164979.1
D2	*Trametes polyzona*	*99%*	JN164979.1
D3	*Trametes polyzona*	*99%*	JN164979.1
D4	*Trametes polyzona*	*99%*	KJ654516.1
D5	*Trametes cf. polyzona*	*98%*	JN164977.1
D6	*Trametes polyzona*	*99%*	JN164980.1
D7	*Trametes polyzona*	*99%*	JN164980.1
D9	*Trametes polyzona*	*99%*	JN164978.1
D11	*Trametes polyzona*	*99%*	KP013053.1
D13	*Trametes polyzona*	*99%*	JX941573.1
E1	*Truncospora macrospora*	*93%*	KC867362.1
E2	*Trametes apiaria*	*94%*	JX941573.1
E3	*Truncospora macrospora*	*93%*	JX941573.1
E4	*Truncospora macrospora*	*93%*	HM136871.1
E5	*Fomes sp.*	*93%*	KF573031.1
F1	*Trametes villosa*	*95%*	KC414233.1
G6	*Trametes tenuis*	*92%*	JN164995.1
G9	*Coriolopsis trogii*	*92%*	JN164970.1
I2	*Trametes villosa*	*95%*	KC848334.1
I3	*Trametes marianna*	*99%*	JQ806418.1
I4	*Trametes ljubarski*	*97%*	HM136871.1
J1	*Fomes sp.*	*94%*	KC867359.1
J2	*Trametes hirta*	*93%*	HM136871.1
J3	*Fomes sp.*	*94%*	KF541332.1
J4	*Fomes sp.*	*94%*	KC867362.1
J6	*Trametes apiaria*	*94%*	KC867359.1
J7	*Trametes hirta*	*93%*	KF541332.1
J8	*Fomes sp.*	*94%*	KF541332.1
J9	*Fomes sp.*	*94%*	KF541332.1
K3	*Trametes sp*	*99%*	KP013021.1
K4	*Trametes ljubarski*	*96%*	JQ806418.1
K5	*Trametes hirsuta*	*93%*	JF439511.1
K6	*Trametes villosa*	*94%*	JN164970.1
M6	*Trametes trogii*	*98%*	HM989941.1
M7	*Coriolopsis caperata*	*99%*	AB158316.1

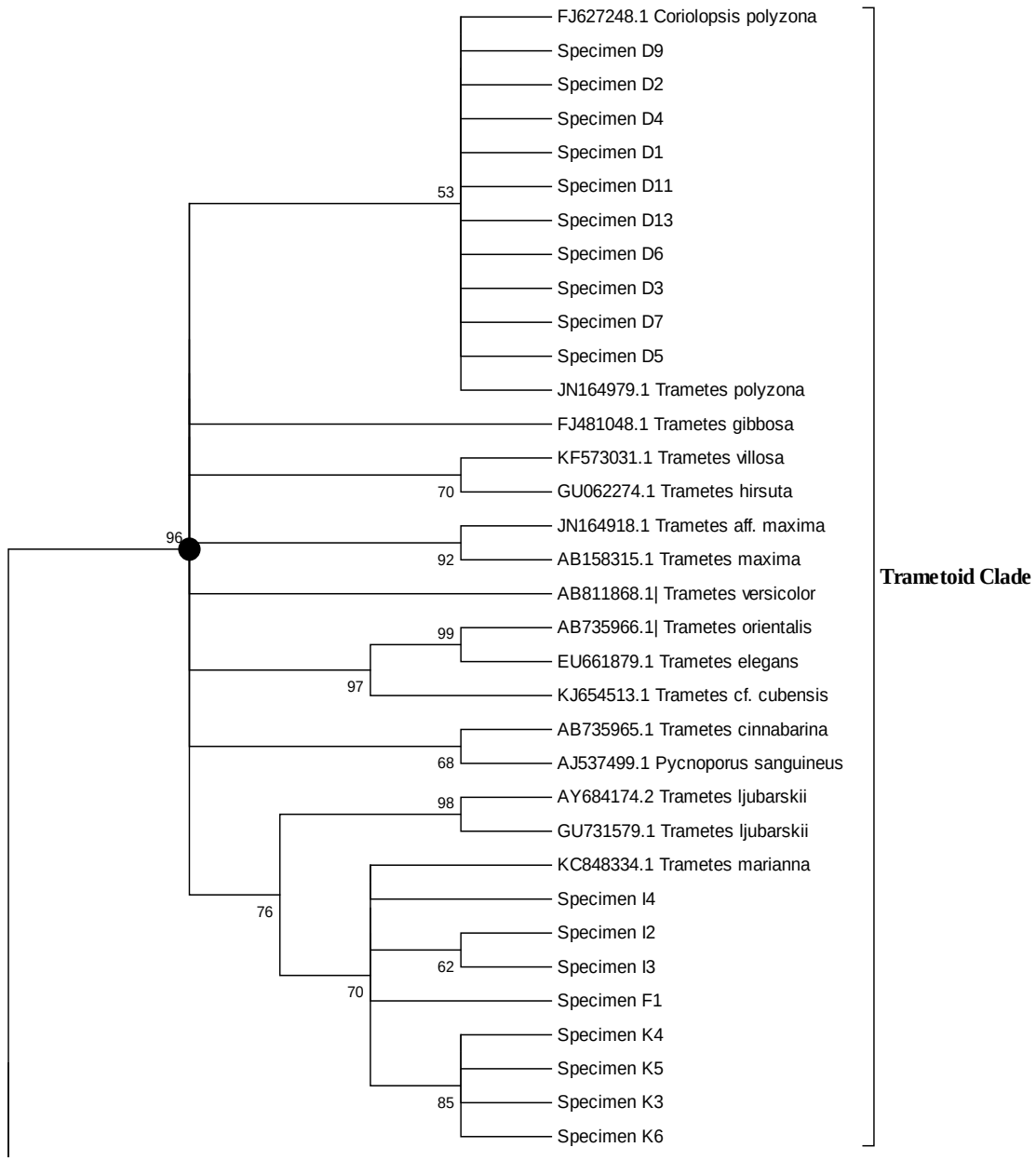

Figure 14. Trametoid clade containing unpublished ITS1 and ITS2 fragment sequences from Northern Namibia and *Trametes* species sequences from GenBank. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.40518603 is shown. The percentage of replicate trees

in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site.

4.5 Ethnomycology of indigenous *Trametes* mushroom in Northern Namibia

Interviews were conducted in Oshana, Ohangwena and Omusati region to gain ethnomycology of the *Trametes* mushroom in Northern Namibia. A total of 62 people were interviewed and 64 % of the total population was from Omusati region, 23 % from Ohangwena and lastly 13 % from Oshana region (Figure 15).

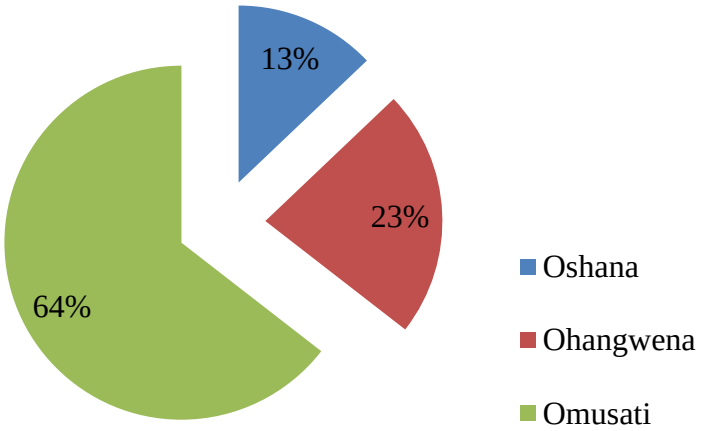

Figure 15. Percentage of responses per region.

For this study, only people who were 25 years or older were interviewed to ensure that reliable information was given. The age group of 60-80 made up the bulk of the respondents interviewed with 55.9 % followed by the 30-39 age groups with 13.6 %. The 40-49 and 50-59 age groups were both represented with 11.9 % and the lowest was in the 25-29 and 80^{+} age groups with only 3.4 % representation (Figure 16).

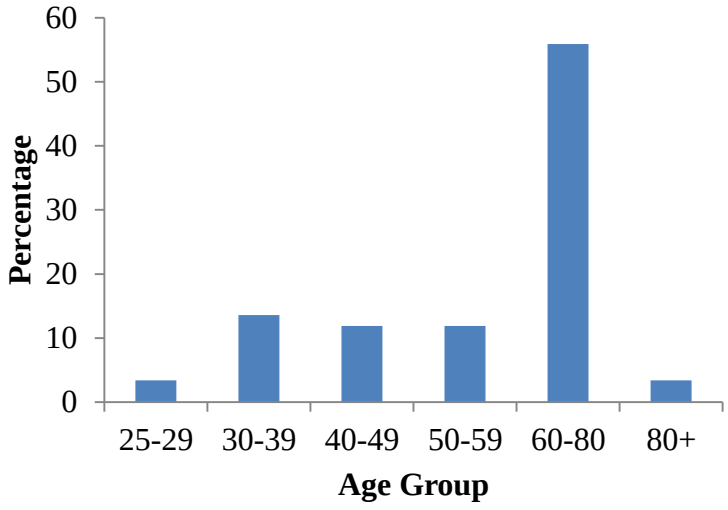

Figure 16. Age group participation in the study.

The *Trametes* mushroom was described to participants before they were asked if they knew about it. A *Trametes* sample was occasionally shown to participants. Figure 17 shows that based on percentage response per region, the respondents from Ohangwena region had more knowledge (92 %) of the *Trametes* mushroom compared to Oshana (63 %) and Omusati region (85 %). However, ANOVA results show that there is no significant difference in *Trametes* mushroom knowledge from the three regions. Of the

participants who knew *Trametes* mushrooms, 76 % used it for medicinal purposes while 24 % had no medicinal application for the mushroom (Figure 18).

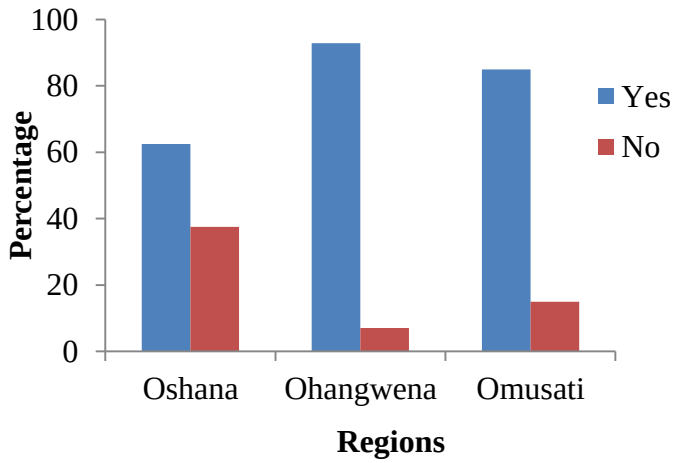

Figure 17. Percentage of people who know the *Trametes* mushroom in Ohangwena, Oshana and Omusati regions.

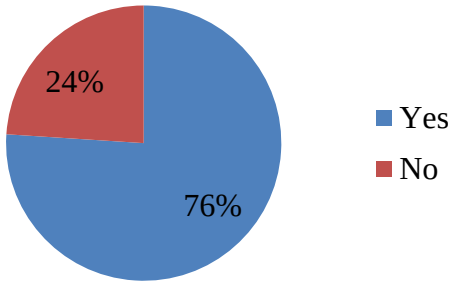

Figure 18. Percentage of people who use *Trametes* mushrooms for medicinal purpose in Ohangwena, Oshana and Omusati regions.

The participants who used *Trametes* mushroom were asked to indicate the different conditions they used it for. A variety of conditions such as shock (23.7 %), calming the bereaved (43.1 %) and children using it as a toy by licking the pores (6.9 %) were recorded (Figure 19). Another use reported for *Trametes* mushrooms include inducing vomiting in cases of mushroom poisoning. During this study, it was discovered that the local people had no specific prescription regarding how often the mushroom should be taken before its health benefits are noticed. Most participants take it daily (40 %), continuously until healed (23 %), or only once (19 %). Some participants (4 %) were not sure how often the mushroom is taken before an illness is healed (Figure 20).

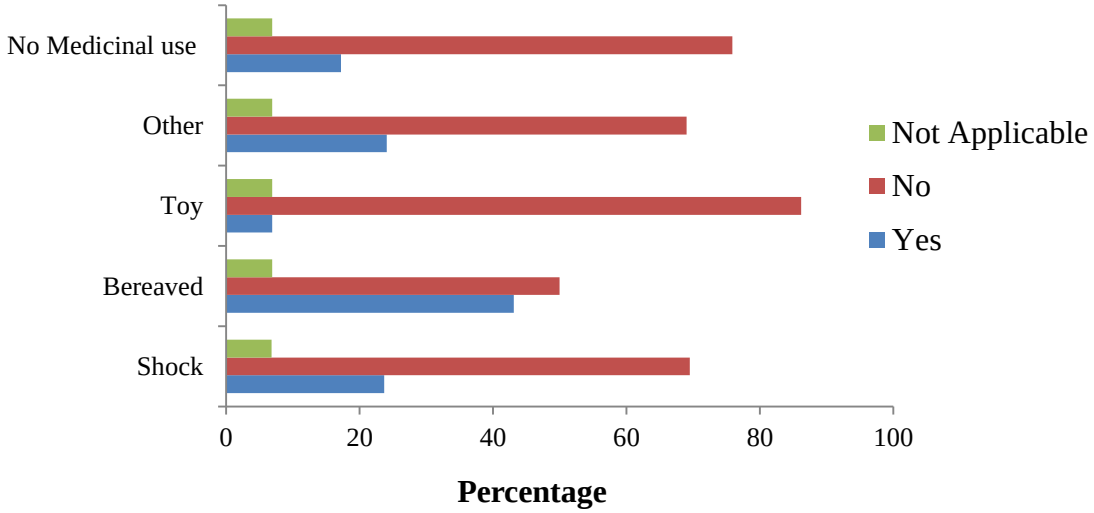

Figure 19. Different uses for *Trametes* mushrooms in Northern Namibia.

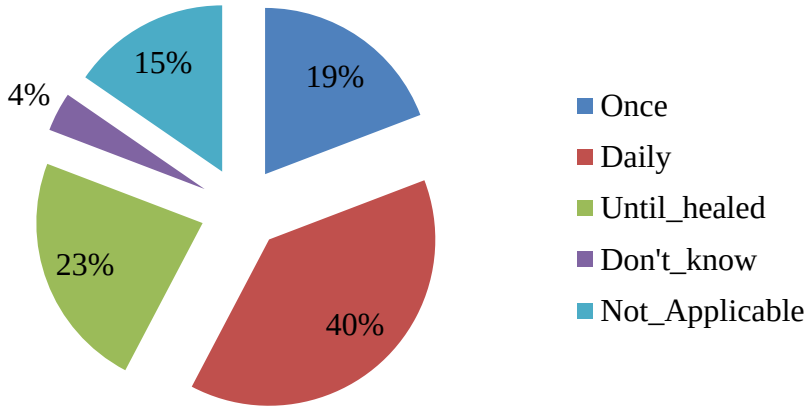

Figure 20. Prescription of the *Trametes* mushroom before improvement or healing is observed.

Participants were further requested to explain how they administer the *Trametes* mushroom for its health benefits. Breaking it into pieces and making a water infusion was the most preferred method (85 %) followed by crushing it into powder (7 %) or roasting and smoking it (2 %). Other methods (3 %) were recorded where pieces of the *Trametes* mushroom are placed into a mourner's cup from which they will take all their drinks during the mourning period (Figure 21).

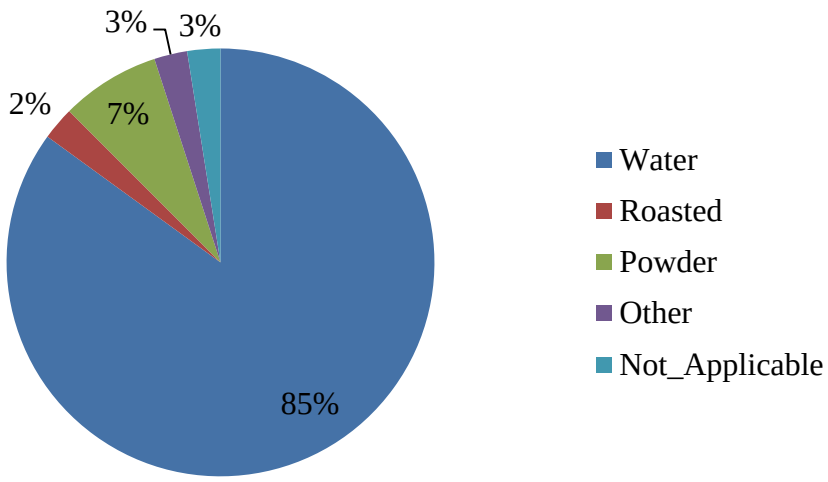

Figure 21. Forms in which *Trametes* mushroom is administered medicinally.

In order to obtain some information about the fruiting season of *Trametes* mushrooms, participants were asked when they harvest the mushroom. *Trametes* mushrooms in Northern Namibia appear or grow during the rainy season when 37 % of people harvest them. Their tough nature however allows the fruit bodies to remain intact on their substrate and 53 % people only harvest them as the need arises (Figure 22). Participants were also inquired about the substrate on which they find *Trametes* growing. As was expected, 84 % of the participants said the common substrate for this mushroom was wood. Surprisingly, 7 % of the participants reported that they found *Trametes* species growing on live trees (Figure 23).

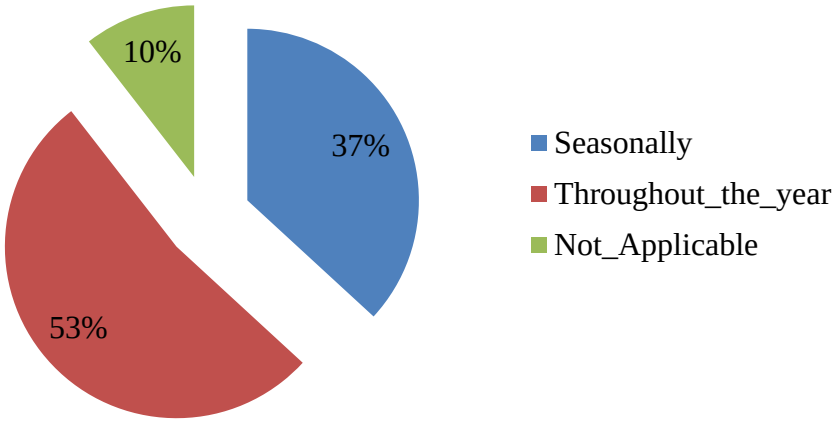

Figure 22. Seasons when *Trametes* mushrooms are harvested in Northern Namibia.

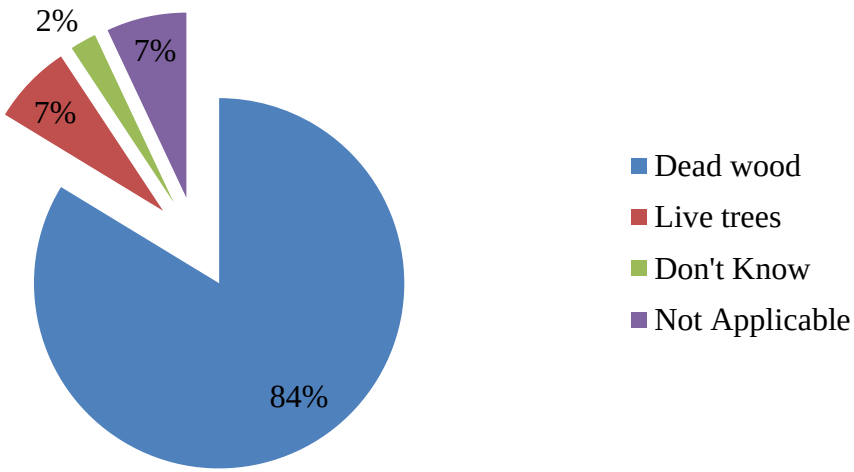

Figure 23. *Trametes* substrates in Northern Namibia.

Mushroom preservation is important to secure its availability during the dry months. Participants were asked how they preserve the *Trametes* mushroom. The majority (48.5 %) prefer to leave them on the host substrate until needed while 33.3 % harvest and dry them before storage. The rest of the participants did not know any preservation methods (9.1 %) (Figure 24).

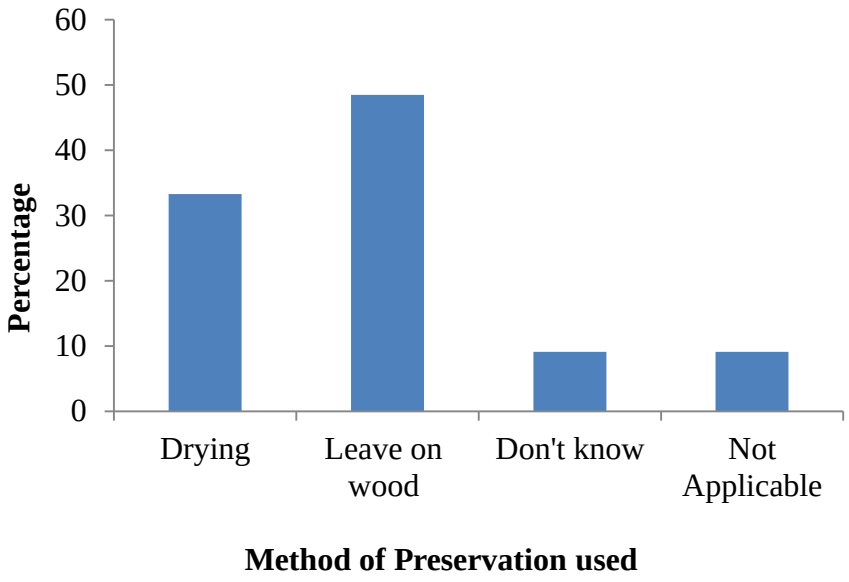

Figure 24. Preservation methods of *Trametes* mushrooms in Northern Namibia.

CHAPTER 5: Discussion

The objective of this study was to confirm the identity of Namibian indigenous *Trametes* species from Northern Namibia for the first time. Another objective was to generate information on the local uses and bioactive compounds of these indigenous *Trametes* species. Mushroom samples collected from Northern Namibia were identified as *Trametes* species according to morphological features such as concentric zones on the basidiocarp and wide pores under the basidiocarp (Van der Westhuizen & Eicker, 1994). However, molecular results obtained in this study showed that not all the collected samples grouped in the core Trametoid clade. Molecular results further showed that the *Trametes* samples from Northern Namibia had 99% identity to *Trametes polyzona* sequences on the NCBI gene databank. TLC visualization reagent tests revealed that flavonoids and alkaloids were present in these indigenous *Trametes* mushrooms. Biological assays further revealed that the DCM extract of indigenous *Trametes* mushrooms from Northern Namibia had bioactivity against *E. coli*, *B. subtilis*, *N. meningitidis* and *A. faecalis*. Finally, ethnomycology surveys administered at Northern Namibia indicated that the local people in the study area have indigenous knowledge about the *Trametes* mushrooms and they use it for medicinal applications.

5.1 Morphological and Molecular Identification

The mushrooms collected in this study presented a morphology characteristic of *Trametes* species although high variation was observed in size and colour of fruit bodies, size and shape of pores, concentric zones and rigidity of mushroom upon breaking or tearing (Van der Westhuizen & Eicker, 1994). Literature shows that the morphological variation observed with the *Trametes* samples in this study is encountered worldwide. According to Zmitrovich et al. (2012) the *Trametes* genus is characterized by wood inhabiting species with a basidiocarp diameter size ranging from small (1-3 cm) to medium (3-10 cm) and large (12 cm or more). Zmitrovich et al. (2012) further elucidated that the *Trametes* species can appear white, cream, tan or brown in colour, with the basidiocarp being tomentose with small erect hairs, villose with sparse hairs or glabrous; smooth and without any hair. They also described pore shape as poroid (round tubular and deep, sometimes elongated pores), hexagonoid (short tubular pores with hexagonal shape), daedaleoid (radially elongated pores mixed with lamellae) and lamellate (radial lamellae). Based on these morphological differences, at least four *Trametes* species were identified from Northern Namibia.

The phylogenetic tree reconstructed in this study showed 8 major clades which were all supported with bootstrap values higher than 50 %. All the samples which had mushroom D morphology were classified as *Trametes* species and grouped in the Trametoid clade

with a bootstrap value of 96 % at the main branch node. Bootstrap values are a proportion of how many times a tree which is being grouped is recovered and are used as a measure of support for a specific clade forming (Soltis & Soltis, 2003). A bootstrap value of 96 % therefore provides a high confidence for the unpublished sequences from this study to be grouped in the Trametoid clade.

Although the mushrooms were positively identified as *Trametes* species using morphologic features, molecular results show that not all the samples collected were indeed *Trametes* species. This demonstrates that although the traditional taxonomy of *Trametes* species was based on morphological features it is not always reliable because these features are affected by nutrient status and growth conditions (Moore, Gange, Gange & Boddy, 2008). According to Aguilar-Trigueros, Powell, Anderson, Antonovics and Rillig (2014), differences in gene sequences of *Trametes* species from Namibia and their counterparts worldwide may be due to the different ecological zones where they are growing. Some species are similar in their morphology such that it is difficult to delineate them based on morphology alone (Yang, Li, Li & Wen, 2011).

It was interesting to observe that samples D1-D9, D11, D13, F1, I2-I4, K3-K6, E2, E5 and J1, J3-J9 were all collected from Ohalushu village in Ohangwena region but they formed separate clades. A possible reason for this could be the high plasticity of fungi which causes individuals to display spatial and temporal variation in their morphology

and physiology (Aguilar-Trigueros et al., 2014). According to Oyetayo (2014), these differences might also occur because of geographical and environmental factors. Geographic factors can cause fungal diversity at a regional level in a radius of 1000-4000 km while environmental factors may cause diversity between species which are within a radius of less than 1000 km (Oyetayo, 2014).

In the phylogenetic tree obtained in this study, Specimen D1-D9, D11, D13 and Specimen F1, I2-I4 and K3-K6 were grouped in the Trametoid clade together with *Trametes* species such as *T. polyzona*, *T. gibbosa*, *T. villosa*, *T. hirsuta*, *T. maxima*, *T. cinnabarina*, *T. cubensis*, *T. orientalis*, *T. elegans* and *T. marianna*. These results are similar to those reported by Welti et al. (2012) and Carlson, Justo and Hibbett, 2014. Welti et al. (2012) reconstructed the phylogeny of the *Trametes* group using Bayesian analysis of ITS1-5.8S-ITS2 and RPB2 to confirm the close relationship between the genera *Trametes*, *Coriolopsis* (*polyzona*) and *Pycnoporus.* One of the most recent and comprehensive work on *Trametes* phylogeny used molecular data from the ribosomal large sub-unit (nLSU) and ITS as well as the RPB1, RPB2 and TEF1-alpha protein coding genes. Similar to the results obtained, the five marker molecular analysis strongly supported a Trametoid clade which includes most *Trametes* species (*T. suaveolens*, *T. versicolor*, *T. maxima*, *T. cubensis*) and *Lenzites*, *Pycnoporus* and *Coriolopsis polyzona* species. Furthermore, the position of *T. trogii* (= *Coriolopsis trogii*) was confirmed to be

outside the Trametoid clade and more closely related to *C. gallica* (Justo & Hibbett, 2011; Tomšovsky et al., 2006).

The genus *Coriolopsis* is currently defined as polyphyletic with type species in the Trametoid clade and two additional lineages in the core polyporoid clade (Carlson, Justo & Hibbet, 2014). This explains why *Trametes trogii* was placed in a clade much further from other *Trametes* species but closer to *Coriolopsis gallica* and *C. trogii* during this study. The results obtained in this study illustrate that morphologic features are not always a reliable way to identify mushroom taxonomy. Mushrooms could look similar in morphology without them being closely related genetically. They can also look different in physical features but be closely related genetically. For example, the *Pycnoporus* mushroom has a red basidiocarp but apart from that, it is morphologically similar to *Trametes* species. Other biochemical characters between the two genera do not differ and molecular analysis of the ribosomal DNA groups the two genera in one clade (Tomšovsky et al., 2006), just as confirmed in this study by grouping *Pycnoporus sanguineus* in the Trametoid clade with *Trametes* species. Based on the phylogenetic tree obtained in this study, the authors support the decision to keep a single generic name of *Trametes* for the Trametoid clade because it allows preservation of the morphological concept of *Trametes*, classification of additional species which may not yet be sampled or analysed and of *Trametes* species using morphological features alone (Justo & Hibbett, 2011). Any other scenario which divides the Trametoid clade deems

extremely difficult, even impossible (Justo & Hibbett, 2011). This is because dividing the Trametoid clade to exclude distant related genera like *Coriolopsis* will make it difficult to classify any new species which are narrowly related to *Trametes* but do not fit in the classification of other genera and clades. The results of this study also lead to ask the question whether narrowly related genera such as *Coriolopsis*, *Coriolus*, *Lenzites* and *Pycnoporus* should be recognized as independent monophyletic genera or whether they should be included in an enlarged *Trametes* genus (Welti et al., 2012). The need for more studies to answer the questions and confusion in *Trametes* taxonomy is highlighted here.

Species delimitation remains a challenge due to the highly variable and sometimes similar morphological features of polypores. Hence, a more precise assessment of the natural selection may be achieved by not only using phylogenetic and morphological methods but also biochemical methods (Lesage-Meessen et al., 2011). Multi-gene datasets have also been used recently to clarify the systematic relationship of fungi (Wu, Yuan, Malysheva, Du & Dai, 2014). The use of high performance liquid chromatographic analysis (HPLC) combined with mass spectrometry (MS) and nuclear magnetic resonance (NMR) is an alternative method which could be used to differentiate among species of the same genus (Dachtler, Glaser, Kohler & Albert, 2001; Ofodile, Attah & Simmonds, 2007). For example the profiles of the phenolics in an extract, which are species-specific, can be used to identify mushrooms (Tibuhwa, 2012). A

similar approach of sequencing the entire genome of *Trametes* species and related genera could be used to solve the confusion regarding their phylogeny.

5.2 Solvent Extractions

Organic and aqueous extract yields obtained from *Trametes* mushrooms in this study was low compared to other mushrooms reported (Tibuhwa, 2012; Awala & Oyetayo, 2015). Awala and Oyetayo (2015) prepared organic extracts of *Trametes* mushrooms and got a yield as high as 2.2 mg/g for the methanol extract, although acetone yield was lower with 1.2 mg/g. The highest yield for this study was from DCM with only 0.09 mg/g obtained. The extract yield of any reaction depends on many factors such as the solvent used, polarity of the solvent and polarity of the mushroom compounds (Tibuhwa, 2012). Additional factors such as pH, length of extraction time, temperature and the chemical composition of the mushroom can also greatly affect the yield obtained (Awala & Oyetayo, 2015). For example, if the *Trametes* samples had high concentration of phenolic compounds, a highly polar solvent like methanol would be able to form strong hydrogen bonds with the phenolic compounds in the mushroom and extracts them (Tibuhwa, 2012). Lastly, the authors take into consideration that the sequential extraction method used in this study, could have lowered the yield from the indigenous *Trametes* mushrooms. With sequential extraction the same mushroom material is exposed to all the different solvents, dividing the total chemical compounds in that mushroom material across all the solvent types used. For the method employed by

Tibuhwa (2012) and Awala and Oyetayo (2015), fresh mushroom material was used each time.

The determination of phenolic secondary metabolites in the *Trametes* mushroom is important because the antioxidant activity of most plant and fungal material is reportedly due to the presence of their phenolic components (Yap et al., 2014). Alkaloids have antimicrobial, antidiarrheal, anthelmintic properties. Coumarins have antimicrobial properties and flavonoids have antimicrobial and antidiarrheal properties (Tiwari, Kumar, Kaur, Kaur & Kaur, 2012). TLC of the methanol extracts obtained in this study indicated the presence of alkaloids and flavonoids. On the contrary, *Trametes lactinea* mushrooms from Nigeria tested negative for alkaloid, although they also had flavonoid content (Awala & Oyetayo, 2015). The difference observed in mycochemical content of Namibian and Nigerian *Trametes* mushrooms is not an unusual occurrence. It is argued that although mushrooms may belong to the same genus or family or even species, differences in mycochemicals produced may be affected by differences in ecological zones and age of the fruiting body of the mushroom, among others (Jha & Tripathi, 2012b; Awala & Oyetayo, 2015).

5.3 Antimicrobial Analysis of indigenous *Trametes* mushrooms

In this study, *N. meningitidis* showed the highest susceptibility to DCM extract of indigenous *Trametes* mushroom with 12 ± 1 mm inhibition zone while *B. subtilis* showed the lowest susceptibility to DCM extract with 9.7 ± 2.08 mm inhibition zone. The DCM extract of Namibian indigenous *Trametes* mushroom exhibited broad spectrum activity against both Gram positive and Gram negative bacteria. Furthermore, the DCM extract showed the strongest activity against *N. meningitidis*, a Gram negative pathogen. Gram negative bacteria have an outer membrane which consists of. These hydrophilic polysaccharide chains act as a barrier to hydrophobic essential oils, enabling the bacteria to degrade foreign molecules entering from outside the cell (Chia and Yap, 2011). These results show the potential of indigenous Namibian *Trametes* mushrooms to be used as source of antibiotics against Gram negative bacteria. This is an encouraging result because Gram negative bacteria are usually highly resistant to antibiotics (Yamaç & Bilgili, 2006). This result is further supported by Awala and Oyetayo (2015) who found that *Trametes lactinea* from Nigeria was more active against Gram negative pathogens in their study.

Apart from DCM, all the four remaining extracts were inactive against the test microorganisms (0.0 ± 0.0 mm inhibition). This was unexpected; especially since TLC reagent tests confirmed the presence of flavonoids in the methanol extract of *Trametes*. It was expected that the methanol extract would also display antimicrobial activity

against some of the bacteria tested because flavonoids are reported to have strong antibacterial activity (Orhana, Özçelikb, Özgenb & Erguna, 2010). There could be various reasons for this apparent lack of bioactivity. Genetic, physiological and environmental factors as well as extraction conditions can influence the chemistry and chemical composition of natural products such as mushrooms. Since the secondary metabolite composition of mushrooms is heavily dependent on its growth conditions and environmental factors such as light, temperature and water availability, these can strongly modulate the biosynthesis of bioactive secondary metabolites in the mushroom (Pani, 2011). It is therefore necessary to fully characterize the composition (and seasonal fluctuations thereof) of mushrooms with wide medicinal use. Especially considering that the different response of a mushroom to its environment may result in a variable presence of natural products to which human can draw on to benefit even if the collection time does not match the balsamic period of the medicinal species (Galasso et al., 2014). It is also common for co-specific isolates of fungi to produce distinct secondary metabolites which might have different antimicrobial activity (Yamaç & Bilgili, 2006). For this reason, it is important to screen different samples or isolates of the same species of mushroom. Therefore, the hexane, ethyl acetate, methanol and hot water extracts of Namibian indigenous *Trametes* mushroom isolates should be recommended for further screening.

It was observed that the DCM extract of Namibian indigenous *Trametes* mushroom did not show any susceptibility against *S. aureus* (0.0 ± 0.0 mm inhibition). This was

contrary to the results obtained by Yamaç and Bilgili (2006) when DCM extract of *Trametes versicolor* was shown to be active against *S. aureus* in disc diffusion assay. It is suggested that purifying or increasing the concentrations of the extracts obtained in this study could improve their performance. This is especially so when considering that commercial antibiotics are effective due to their high degree of purity and reduced molecular sizes, resulting in a more concentrated product with higher solubility (Awala & Oyetayo, 2015).

The antimicrobial activity of Namibian indigenous *Trametes* mushrooms is generally in general agreement with previous studies. The count of *Clostridium, Staphylococcus* and *Enterococcus* species were reduced when incubated in *Trametes* PSP extract by Yu, Liu, Mukherjee and Newburg (2013). It was hypothesized that *Trametes* mushrooms mechanism for antimicrobial activity is effected directly upon contact, indirectly, or both ways, leading to a prebiotic effect when ingested by humans. The prebiotic effect is due to the β-glucans in PSP which are not easily digested by the intestinal enzymes of humans. They remain in the gut until they are used up by colonic microbiota or excreted in the stools. Some of these indigestible dietary glycans then modify the intestinal bacteria in humans and modulate the immune system (Yu et al., 2013). Literature further stated that *Trametes versicolor* has biological activity against HIV (Aisya, Naveen, Pravalika, Haritha & Anusha, 2010; Lindequist et al., 2005), *Aspergillus niger*, *Candida albicans*, *E. coli* and *Streptococcus pneumonia* (Aisya et al., 2010). A similar study which screened Nigerian *Trametes cingulata, T. mariana,* and *Daedalea quercina* also

reported activity against *Bacillus subtilis* and *Pseudomonas syringae* (Ofodile et al., 2007).

In a study by Yamaç and Bilgili (2006) the antimicrobial activity of 20 mushroom species was evaluated, using fruit bodies and mycelial cultures. The *Trametes versicolor* fruitbodies used in that study only yielded activity for *S. aureus* (DCM and acetone extract) and *Pseudomonas aeruginosa* (acetone extract). However the mycelial cultures showed activity against *E.coli*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *S. aureus*, *Streptococcus epidermidis* for aqueous extract and *E.coli*, *E. aerogenes*, *S. typhimurium*, *S. aureus*, *S. epidermidis* and *B. subtilis* for organic extracts (Yamaç and Bilgili, 2006). The above results illustrate an area of great discussion in the medicinal mushroom field. It is not known whether the bioactivity of a mushroom depends on a single compound alone or whether it is a result of synergy between different compounds. It is also not known whether it is more effective to use fruiting bodies, submerged mycelia cultures or crude extracts and isolated fractions from the mushroom (Wasser, 2011). There is therefore a need to provide sufficient standards for the recommended use of medicinal mushrooms and this can only be made available by thorough studies and characterization of indigenous medicinal mushrooms (Wasser, 2011).

5.4 Ethnomycology of *Trametes* mushrooms in Northern Namibia

A total of 62 people were interviewed and 64 % of the total population was from Omusati region, 23 % from Ohangwena and only 13 % from Oshana region. Omusati region was the largest of the three with a total surface area of 26 551 km^2, double that of Ohangwena region (10 706 km^2) and lastly Oshana with the smallest total surface area of 8 647 km^2 (Namibia Planning Commission (NPC), 2012). Due to the large area mentioned above, the study area did not include the total area of the three regions, rather, only part of it was sampled. Furthermore, the results from ethnomycology surveys are from interviewed persons only and may not depict a full representation of the whole population. Ohangwena region has a population size of 245 100 people and Omusati region, which had the highest percentage participation in this study has a population size of 242 900 people. Oshana region population size (174 900) was half that of Ohangwena and Omusati regions (NPC, 2012), thus reflected by the lower representation of only 23 % in this study. The representation of participants from the three regions was not surprising. It was observed that regions which had a bigger surface area and a bigger population size seemed to have higher participation in the study. This could be because respondents were identified by walking/driving from house to house at traditional homesteads and interviewing readily available respondent. People from Northern Namibia who were not interviewed in this study might have had different ethnomycology of the indigenous *Trametes* species.

To ensure that the information obtained was reliable, only people who were 25 years or older were interviewed. The age group of 60-80 years made up the bulk of the respondents interviewed with 55.9 % followed by the 30-39 age groups with 13.6 %. The 40-49 and 50-59 years age groups were both represented with 11.9 % and the lowest was in the 25-29 and 80^{+} age groups with only 3.4 % representation. National census studies have revealed that the population in rural areas generally consists of young people (0-14 years) and senior citizens (60^{+} years) while the economically active group (15-59 years) lives and works in urban areas (National Statistics Agency (NSA), 2013; Indongo, 2015). This could be the reason why the highest participation was in the older group of pensioners. In fact, the highest represented (55.9 %) age group was of participants 60 years and older while the lowest participation (3.4 %) was in the 25-29 age groups. It can be said that because the majority of respondents were older, the information obtained from the surveys is considered more reliable. In addition, indigenous knowledge is usually found in the elderly people of a community (Ekandjo & Chimwamurombe, 2012). Therefore, these results also show that the ethnomycology of the local people in Northern Namibia was held mostly by the older people.

The *Trametes* mushroom was described and shown to participants before they were asked if they knew it. The mushroom was identified positively by 84 % of the participants but 16 % were not familiar with it. Of the participants who knew *Trametes* mushrooms, 76 % used it for medicinal purposes while 24 % had no medicinal application for the mushroom. The study revealed that although the local people were

aware of the mushroom, only some knew about its medicinal quality and used it. It appears that local people do not always know about the medicinal applications of mushrooms. This observation was also reported about a different medicinal mushroom, called *Ganoderma*, in the North Eastern parts of Namibia. Similar to the *Trametes* mushroom, the local people in Northeast Namibia knew about it but they did not use it for medicinal applications (Ekandjo & Chimwamurombe, 2012). This study also revealed that although the local people were aware of the mushroom, only some knew about its medicinal quality and used it. The need for studies such as the current one is clearly highlighted here. Literature has shown that indigenous knowledge of African people is passed on orally from older generation to younger generation (Oyetayo, 2011). This valuable information may be lost if not studied and well documented in written form. There is also a need for people to be made aware of medicinal mushrooms which are readily available in their local areas, in order to maintain good health and to keep the indigenous knowledge.

The participants of this study pointed out a number of conditions which can be treated with indigenous *Trametes* species. A variety of conditions such as shock (23.7 %), calming the bereaved (43.1 %) and children using it as a toy by licking the pores (6.9 %) were recorded. Other uses reported for *Trametes* mushrooms in this study include inducing vomiting in cases of mushroom poisoning and using the mushroom to treat lung disease in cows. Similarly, *Ganoderma*, a different type of medicinal mushroom

was reportedly used to calm stressed people and to treat lung disease in cows at the North and North-Eastern parts of Namibia (Kadhila-Muandingi & Chimwamurombe, 2012). In contrast, *Trametes* mushrooms worldwide are most popular for their anticancer properties (Standish et al., 2008; Prasad, Rathore, Sharma & Yadav, 2015). However, no report was made of using *Trametes* mushroom to treat cancer or cancer related symptoms such as tumors and lumps in Northern Namibia. This was surprising because *Trametes* species are considered highly in adjuvant cancer therapy in other parts of the world (Patel & Goyal, 2012).

Respondents who took part in this study reported that *Trametes* mushrooms are used to calm the bereaved or people who are in shock in Northern Namibia. In a study by Marsh et al. (2014), they reported that crude aqueous extract of the plant *Scutellaria lateriflora* had been used as a nerve tonic, sedative, and anticonvulsant for centuries by Europeans and Native Americans. The plant's anxiolytic and mood improving effects were thought to occur through flavonoid and amino acid activity. Additional in vitro studies have shown that some flavones such as wogonin do indeed act on different brain receptors, resulting in mood enhancement (Marsh et al., 2014). This is interesting because the TLC visualization reagent test in this study confirmed the presence of flavonoids in the Namibian indigenous *Trametes* mushrooms. The calming effect experienced by the local people in Northern Namibia when they ingest indigenous *Trametes* mushrooms might be caused by these flavonoids. Further characterization of *Trametes* flavonoids is needed to

explore their anxiolytic properties. What more, the biological activities of the flavonoid polyphenols extracted from *S. lateriflora* were also reported to have anti-cancer properties (Marsh et al., 2014). This could explain the wide use of *Trametes* metabolites in cancer therapy.

Trametes mushrooms grow on wood and are commonly found on the poles used to build homesteads in Northern Namibia. In this study, Ohangwena region was found to have more fruiting bodies of indigenous *Trametes* mushrooms compared to Oshana, Omusati and Oshikoto regions. Similarly, Kadhila-Muandingi (2010), reported that more indigenous *Ganoderma* mushrooms were found growing in Ohangwena region compared to Oshana region. The higher population of vegetation in Ohangwena region provides more substrate for wood inhabiting fungi like *Trametes* and *Ganoderma* to grow on, unlike in the other regions where vegetation is more scarce (Kadhila-Muandingi, 2010). Literature also reports that Ohangwena region is closer to the denser tree vegetation and rivers such as the Okavango, receiving relatively more annual precipitation compared to Omusati and Oshana regions allowing for better mushroom growth (Hassler et al., 2010; Rohde & Hoffman, 2012).

In addition, participants explained that they administered the medicinal mushroom in different forms. Breaking it into pieces and making a water infusion was the most preferred method (85 %) followed by crushing it into powder (7 %) or roasting and

smoking it (2 %). Other methods (3 %) were recorded where pieces of the *Trametes* mushroom are placed into a bereaved person's cup from which they will take all their drinks during the mourning period. It was found that soaking the mushroom in water, crushing to powder and roasting are the most common methods in a traditional setting to administer mushrooms which are too tough to eat, according to Ekandjo & Chimwamurombe (2012).

There was no specific prescription regarding how often the mushroom should be taken before its health benefits are noticed. Most participants take it daily (40 %), continuously until healed (23 %), or only once (19 %). Some participants did not know how often the mushroom should be taken before relief from symptoms (4 %). According to Wasser (2011), there is a similar problem in the commercial medicinal mushroom market where the dosage of formulations prepared from medicinal mushrooms has not been established. It has also not been established whether these formulations are safe for pregnant or lactating mothers, neither has the duration of administration been determined. This area clearly needs more dedicated study to provide sufficient safety standards and recommendations for use of medicinal mushroom formulations (Wasser, 2011).

Respondents also gave some information about the fruiting season of *Trametes* mushrooms in Northern Namibia. *Trametes* mushrooms in Northern Namibia appear or grow during the rainy season when 37 % of people harvest them. Their tough nature however allows the fruit bodies to remain intact on their substrate and 53 % people harvest them throughout the year as the need arises. Early rains in Namibia start from October to November, stretching to the full rain season in January and sometimes extending as far as April (Turner, Versfeld, Kilian & Getz, 2012). It can therefore be expected that new *Trametes* fruiting bodies appear between October and April when the rains have provided sufficient moisture.

The majority (48.5 %) of people who took part in this study preferred to leave the *Trametes* mushrooms on the host substrate until when they would need it while 33.3 % preferred harvest and dry them before storing. The rest of the participants were not aware of any preservation methods (9.1 %). As was expected, 84 % of the participants said the common substrate for this mushroom was wood. Surprisingly, 7 % of the participants reported that they found *Trametes* species growing on live trees. A search in literature revealed that although a mushroom normally grows on wood it can be found growing on live trees occasionally (Ekandjo & Chimwamurombe, 2012).

CHAPTER 6: Conclusions

Results addressed three main hypotheses. The first hypothesis stated that the *Trametes* species from Northern Namibia all belonged to the main Trametoid clade. A phylogenetic tree constructed with sequences of indigenous Namibian *Trametes* species formed 8 major clades instead of only one. Furthermore, only the samples which had a tan to brown coloured basidiocarp with fine pores were classified as *Trametes* species and grouped in the Trametoid clade. These were identified as *T. polyzona, T. gibbosa, T. villosa, T. hirsuta, T. maxima, T. cinnabarina, T. cubensis, T. orientalis, T. elegans* and *T. marianna*. All the other samples were grouped in separate clades with *Hexagonia, Coriolopsis trogii* and *Fomes* species. Therefore, not all the *Trametes* species from Northern Namibia belong to the main Trametoid clade.

The second hypothesis stated that the local people of Northern Namibia had different applications for *Trametes* species due to differences in culture and therefore ethnomycology. Results from this study showed that there is no significant difference (at $\alpha = 0.05$) in *Trametes* ethnomycology of Ohangwena, Oshana and Omusati regions. Various applications of indigenous *Trametes* mushrooms for medicinal purposes were also determined.

Lastly, the third hypothesis stated that *Trametes* species from Northern Namibia had varying alkaloids and other mycochemicals due to differences in the age of fruiting bodies and environmental factors such as water availability, substrates and temperature. Results of mycochemical and antimicrobial tests in this study have shown that this is true. Namibian indigenous *Trametes* species' mycochemical profile varies from mycochemical profiles of *Trametes* species from other regions of the world. In this study, *Trametes* species showed presence of alkaloids and flavonoids.

It is concluded that in this study, the identity of Namibian indigenous *Trametes* species from Northern Namibia was confirmed for the first time. Information about the local uses and bioactive compounds of these indigenous *Trametes* species was also generated.

CHAPTER 7: Recommendations

There is a need for more precise species delimitation of indigenous *Trametes* mushrooms in Northern Namibia. This can be achieved by combination of different techniques using not only phylogenetic and morphological but also biochemical methods. Multi-gene datasets sequencing the entire genome of *Trametes* species and related genera and a chemotaxonomic approach are some of the recommended methods that can be used to clarify the systematic relationship of *Trametes* species and closely related genera.

It is also recommended to screen different samples or isolates of the indigenous *Trametes* mushroom in Namibia in order to get an inclusive picture of its mycochemical content and antimicrobial activity. Solvent types and extraction methods, as well as using mycelial culture instead of a mature mushroom fruit body can have an effect on antimicrobial activity detected. It is thus recommended that further studies be done to investigate the mycochemical content and antimicrobial activity of these indigenous mushrooms. Further recommendations are to perform GC-MS analysis of indigenous *Trametes* extracts obtained in this study in order to determine and identify volatile compounds present. This approach could cut short the lengthy process of mycochemical and antibiotic screening, leading to the identification of compounds which are known to be important medicinally. An additional recommendation is to investigate the anticancer potential of indigenous Namibian *Trametes* mushrooms. Although no record was made

of using this indigenous mushroom for cancer related symptoms in this study, *Trametes* mushrooms worldwide are highly valued for their anticancer properties.

Final recommendations are to disseminate information on *Trametes* species in order to educate communities in Namibia, especially Northern Namibia about the medicinal value of *Trametes* mushrooms. This might lead to improved health if used regularly and lead to income generation if cultivated, harvested and sold.

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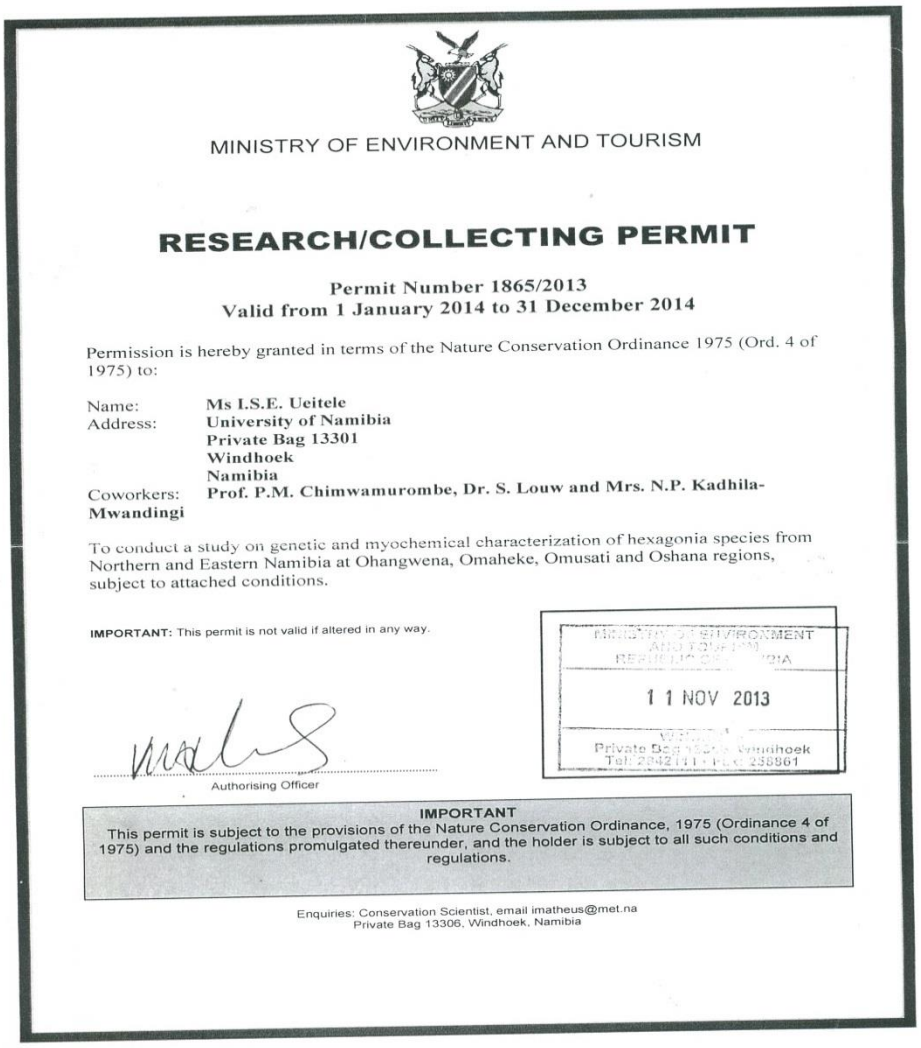
APPENDICES

Appendix 1: Research/ Collecting permit from the Ministry of Environment.



MINISTRY OF ENVIRONMENT AND TOURISM

RESEARCH/COLLECTING PERMIT

Permit Number 1865/2013
Valid from 1 January 2014 to 31 December 2014

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:

Name: **Ms I.S.E. Ueitele**
Address: **University of Namibia**
Private Bag 13301
Windhoek
Namibia
Coworkers: **Prof. P.M. Chimwamurombe, Dr. S. Louw and Mrs. N.P. Kadhila-Mwandingi**

To conduct a study on genetic and myochemical characterization of hexagonia species from Northern and Eastern Namibia at Ohangwena, Omaheke, Omusati and Oshana regions, subject to attached conditions.

IMPORTANT: This permit is not valid if altered in any way.

MINISTRY OF ENVIRONMENT
11 NOV 2013
Private Bag 13306 Windhoek
Tel: 284211 FAX: 258861

Authorising Officer

IMPORTANT
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.

Enquiries: Conservation Scientist, email imatheus@met.na
Private Bag 13306, Windhoek, Namibia

Appendix 2: Questionnaire

Questionnaire: Local Knowledge of Mushrooms in Northern Namibia

Age: …… **Village**:……
Region:…… **Gender**:……..

1. Do you know mushrooms?
   Yes…… No…….
2. What type of mushroom?
3. Do you eat mushrooms?
   Yes……. No……
4. Which type of mushrooms do you eat?
5. How often are mushrooms collected?
6. Who collects them?
7. Do you collect mushrooms for sale?
   Yes…….. No……
8. Do you just collect for own use?
   Yes……. No…….

9. Do you use mushrooms for any purposes other than food?
   Yes…….. No…….
10. Do you use mushrooms for medicinal purposes?
   Yes…… No…….

11. Which mushrooms do you use for medicinal purpose?

12. Do you know this mushroom? (*Trametes* mushrooms will be shown)
   Yes…… No……..

13. What do you use it for?

14. Do you use it for treatment of diseases?
   Yes…… No……..

15. Which diseases do you use it for?

16. When used for treatment, in what form do you use the mushroom? (E.g. powder, in water)

17. In what form do you use the other mushrooms?

18. How often does one need to use *Trametes* before they get healed?

19. How often does one need to use the other mushroom before they get healed?

20. In which months do you collect mushrooms?

21. In which months do you harvest *Trametes?*

22. What do you find *Trametes* growing on? E.g. wood, termite hills, trees

23. What do you find the other mushrooms growing on? E.g. termite hills, trees, in the field

24. Do the animals eat *Trametes*?

Yes….. No……

25. Do animals eat the other mushrooms?
Yes….. No……

26. How do you preserve *Trametes*? E.g. drying, omavanda

27. How do you preserve the other mushrooms? E.g. drying, omavanda

28. Which other mushrooms do you use for medicinal purposes?

29. Where did you learn about mushrooms? E.g. Mother, Father, while herding cattle

30. Do you know someone else who knows about mushrooms?

Yes……. No……..

31. Anything else you want to add?
Yes…… No……..

Appendix 3: Basic Local Alignments of indigenous *Trametes* species

Trametes trogii isolate TEM H2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Sequence ID: gb|HM989941.1|Length: 740Number of Matches: 1
Related Information

Score	Expect	Identities	Gaps	Strand	Frame
416 bits(225)	7e-113()	242/250(97%)	2/250(0%)	Plus/Minus	

Features:

```
Query  1
CGTTACATCGCAATACACATTCTGATACTGAAGCGTTTGTAATAAAACGTAGGCACGGTC  60
           |||||||||||||||||||||||||||||||||||||||| ||| ||||||||||||
Sbjct  277
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Query  61
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           ||||||||||||  |||||||||| |||||||||
|||||||||||||||||||||||||
Sbjct  217  AACGCCTCGCCG--
TAAACGACGCGACGCCTTCCGATACCCACAGTAAGTGCACAGGTGT  160

Query  121
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           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
|||||||
Sbjct  159
AGAGTGGATGAGCAGGGCGTGCACATGCCTCCGGAGAGGCCAGCTACAACCCGTTTCAAA  100

Query  181
ACTCGATAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCC  240

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99
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Query  241  TCTAAATGAC  250
           ||||||||||
Sbjct  39   TCTAAATGAC  30
```

Trametes trogii isolate TEM H2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|HM989941.1|Length: 740Number of Matches: 1

Score	Expect	Identities	Gaps	Strand	Frame
399 bits(216)	7e-108()	239/250(96%)	2/250(0%)	Plus/Minus	

Features:

```
Query  1
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            ||||||||||||||||||||||||||||||||||||||||||||||||     ||||||||||
Sbjct  277
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Query  61
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|||||||||||||||||||||||||
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|||||||
Sbjct  159
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  39   TCTAAATGAC  30
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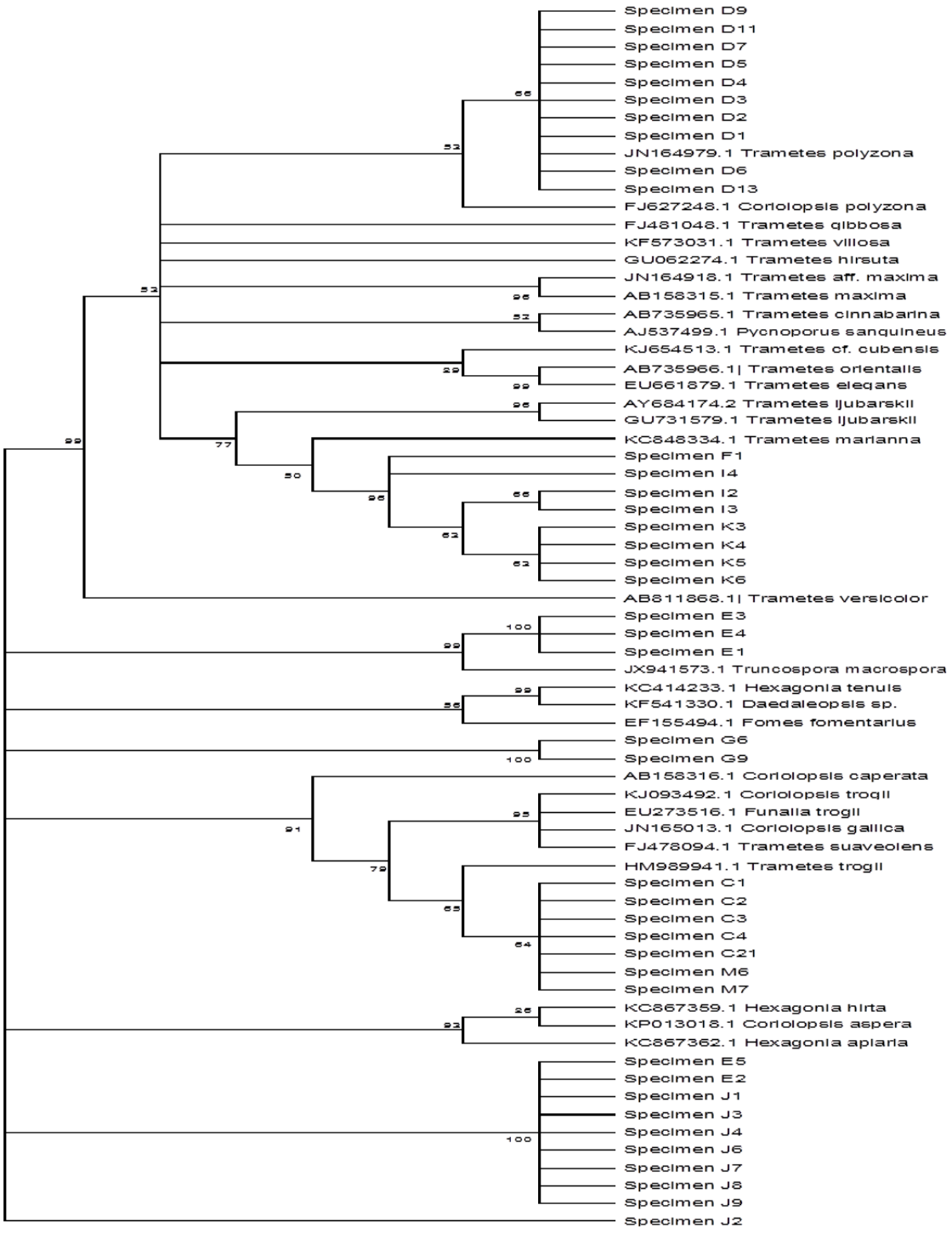
Appendix 4: Evolutionary relationships of taxa.



The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.40518603 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 69 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 162 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Appendix 5: Preparation of Reagents

1 % Ethanolic Aluminium Chloride

0.2 g aluminium chloride was dissolved in 20 ml 99 % ethanol.

Wagner's Reagent

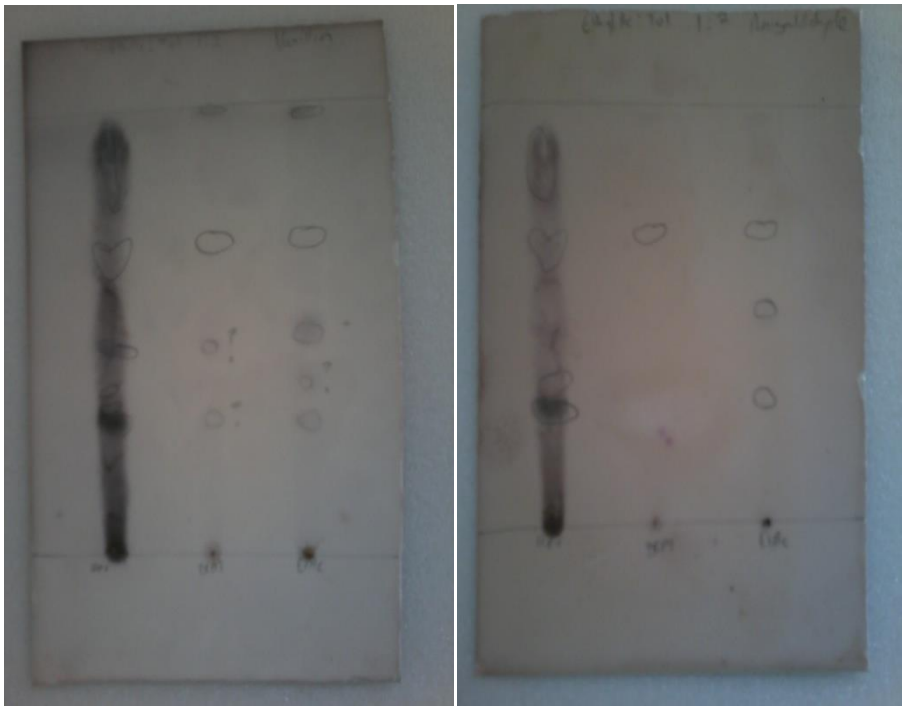
0.2 g iodine and 1.2 g Potassium Iodide were dissolved in 20 ml distilled water.

1 % methanolic $AlCl_3$

0.2 g aluminium chloride was dissolved in 20 ml 99 % methanol.

10 % Methanolic Potassium hydroxide

2 g KOH was dissolved in 20 ml methanol



Trametes chromatograms in vanillin test (Left) and anisaldehyde test (Right).

Appendix 6: Analysis of Variance

The responses from the interviews on *Trametes* ethnomycology at Ohangwena, Oshana and Omusati regions were coded so that **Yes = 1** and **No = 0**.

The results of ANOVA statistical test at $\alpha = 0.05$.

Source of Variation	**Sum of squares**	**d.f.**	**Mean squares**	**F**
between	0.4835	2	0.2418	
error	7.904	59	0.134	
total	8.387	61		1.805

The probability of this result, assuming the null hypothesis, is 0.17 (p=0.17).

$F_{crit} = 3.15312326$

H_o: $\mu_1 = \mu_2 = \mu_3$

H_a: At least two of the means are different.

p>0.05 therefore, fail to reject null hypothesis. There is no significant difference in *Trametes* mushroom knowledge amongst Ohangwena, Oshana and Omusati region.