GENETIC DIVERSITY OF *GANODERMA* SPECIES IN THE NORTH-EASTERN PARTS OF NAMIBIA

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Abstract

The genus *Ganoderma* Karst. (1881) is the largest and most complex genus of polypore fungi, characterized by the presence of pores instead of the gills on the lower side of the fruiting body. The colonisation of *Ganoderma* species on trees is noticed by the appearance of their cap shaped, hard, crusty and shiny fruiting bodies. Ganoderma extract have been used for centuries in Asia's traditional medicine and to date *Ganoderma* mushrooms are an important source of modern medicinal and neutriceutical products in Asia. The objective of this study was to determine the genetic diversity among *Ganoderma* species, the traditional uses and natural hosts of *Ganoderma* species in the north-eastern part of Namibia. This study was carried out in two regions of the north-eastern part of Namibia; Kavango and Caprivi region. Face to face interviews were used to collect data about the uses. A total of 89 *Ganoderma* samples where used for PCR amplification. The genetic diversity was determined by using three different random amplified microsatellites namely ACA, CGA and CCA. Shannon-Weiner Index of diversity, Cluster Analysis (CA) and Principal Coordinate of Analysis (PCoA) were the three numerical classification methods used to analyse RAMS data in this study. All methods revealed existence of high genetic variation among *Ganoderma* isolates in the north-eastern parts of Namibia. CA exhibited 10 different clusters using 3 primers combination, while the first two principal coordinates of PCoA indicated Eigen values of 63.42 and 46.45 with a total variation of 12.20% and 8.93%. Shannon-Weiner Index of diversity indicated 2.16 total genetic diversity for *Ganoderma* in the north eastern parts of Namibia. Thirteen natural hosts of *Ganoderma* species were identified in both Kavango and Caprivi region. The most common host was *Colosphermum mopane* (18%) and the least common host were *Ochna pulchra* and *Grewia bicolor* both accounted for 1% of the total samples. Finally, three different uses of *Ganoderma* were identified mainly strengthening of infant bones (19%), immunity boost (13%) and avert of nose bleeding (4%). However 64% of the respondents did not know any use of *Ganoderma*.

Key words: *Ganoderma*, Genetic diversity, Kavango region, Caprivi region, host and RAMS.

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Declaration

I Lempie Kashinasha Ekandjo 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.

Signature..... Date.....

Dedication

This thesis is dedicated to my mother Mrs Aino Taanyanda and my grand mother Viivi Nangombe Taanyanda who have always supported me, encouraged me to do my best.

Abbreviations

ACA - 5` BDB $(ACA)_5$

AFLP - Amplified Fragments Length Polymorphism

CA - Cluster Analysis

CCA - 5`DD$(CCA)_5$

CGA - 5'DHB$(CGA)_5$

DNA - Deoxyribonucleic Acid

GPS - Global Positioning System

HCA - Hierarchical Cluster Analysis

NBRI - National Botanical Research Institute

PCoA - Principal Coordinate of Analysis

PCR - Polymerase Chain Reaction

RAMS - Random Amplified Microsatellites

RAPD - Randomly Amplified Polymorphism

RFLP - Restriction Fragment Length Polymorphism

SNP - Single Nucleotide Polymorphism

SRAP - Sequence Related Amplified Polymorphism

SSCP - Single Strand Confirmational Polymorphism

TBE - Tris Borate EDTA

UNAM - University of Namibia

UPGMA -Unweighted Pair Group Method with Arithmetic Mean

ZERI - Zero Emissions Research Initiatives

ZR - Zymo Research

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CHAPTER 1

1. General introduction

Ganoderma Karst. (1881) is a large complex genus of polypore fungi (Buchanan, 2001). Polypore fungi are characterized by the presence of pores instead of the gills on the lower side of the fruiting body (Englebrecht and Volk, 2005). The Genus *Ganoderma* is a member of the domain Eukarya; kingdom Fungi; Phylum Basidiomycota; Class Basidiomycetes (higher fungi); Order Polyporales and Family Ganodermataceae (Schwarze and Ferner, 2003). Schwarze and Ferner (2003) further indicated that family Ganodermataceae has 5 genera including genus *Ganoderma*. According to Chang et al. (1996) the genus *Ganoderma* has two subgenus based on variations of its structure, the *Elfvingia* (non laccate species) and subgenus *Ganoderma* under which laccate species belong. Laccate means the shiny upper surface of the *Ganoderma* cap and vice versa (Zheng et al., 2007). *Ganoderma* mushrooms are believed to have a wide geographical distribution worldwide on a wide range of host including; hardwoods, conifers, bamboos and palms (Buchanan, 2001). The genus *Ganoderma* has a very complex taxonomy and consists of about 250 species worldwide (Buchanan, 2001). As a result of this sometimes there are multiple names for a single species within this genus.

There are three levels of biodiversity. These are genetic diversity, species diversity and ecosystem diversity (Campbell and Reece, 2005). This study focused on Genetic diversity as a measure of biodiversity in the genus *Ganoderma*. Genetic diversity refers to a property of a community of organisms of a certain species, in which

members of the community have variations in their chromosomes due to large number of slightly dissimilar ancestors (Huston, 1994). Genetic diversity does not only apply to individuals within a population, but it can also be applied to different populations that are associated with similar environmental conditions (Campbell and Reece, 2005). As result of this, the community in general become more resistant to diseases or to changing ecological conditions. However in most complex genus like *Ganoderma*, genetic diversity is usually genetic diversity analyses have been done to analyse the variation or relation between species through out the entire genus (Zheng et al., 2007; Miller et al., 1999). This is mainly done because *Ganoderma* species exhibit so much similarity and they are hard to distinguish most of the time, except when genetic sequences are analysed to assist in identification of species (Zheng et al., 2007).

A population is regarded as genetically diverse if a substantial proportion of the genes are polymorphic. A polymorphic gene is one for which the most common allele has a frequency of less than 0.95 (Tan et al., 2006). Genetic diversity is usually expressed in terms of percentage of genes that are polymorphic and or are heterozygous. This heritable variation within populations is created, maintained and enhanced by evolutionary or selective forces. Chang et al. (1996) stated that, both macroscopic and microscopic characters have been used in the past to distinguish species within genus *Ganoderma*. They further indicated that some characters such as basidiocarp shape, basidiospore size and context colour are highly influenced by environmental factors and therefore vary in different environments. Analysis of

genetic diversity is very crucial for the exploration of the medicinal value of *Ganoderma* species. In this research DNA analysis employing microsatellites technique was used to determine genetic variations and set up a system for identification between different species of the genus *Ganoderma.*

1.1 Problem statement and Justification

Ganoderma mushrooms are used in China as a source of important raw materials in traditional medicine (Sun et al., 2006). Several researchers indicated important biological activities and high medicinal values of *Ganoderma* extracts such as immunomodulatory action, antitumor, antimicrobial activity and it also has cardiovascular effects (Sun et al., 2006; Chen and Miles, 1996; Mshigeni and Chang, 2001). Namibia is one the countries that import *Ganoderma* products from Asia, beside the fact that there are also indigenous *Ganoderma* mushrooms in Namibia. This is because there is no clarity on which species of this genus exist in Namibia and how diverse are the Namibian *Ganoderma*. This information is very important for determining if Namibia has specific *Ganoderma* species that are of medicinal importance and to formulate conservation measures to protect this medicinally valuable genus before it becomes locally extinct as a result of over exploitation by locals. Lastly, better understanding of the diversity and taxonomy of this Genus would be a starting point for the government to formulate disciplines for exploitation of these mushrooms at a national level.

This study was designed to determine the genetic variation between different species of the genus *Ganoderma.* Determination of genetic variation between different species is very crucial for conservation purposes of this genus. When genetic diversity becomes low at many genes within species, the species becomes increasingly at risk of extinction. This is because the individuals have nearly one form of information and are less likely to adapt to new environment especially if there is occurrence of environmental disaster. Populations that have very low genetic diversity are also at greater risks of extinction and therefore require immediate conservational measures. Therefore, quantifying the genetic diversity between *Ganoderma* species plays a major role in the conservation of this important genus.

Furthermore, the identification of different natural hosts on which *Ganoderma* species grow in the northern part of Namibia will reveal important information that will help people to harvest *Ganoderma* species in Namibia and anywhere else in the world. This is mainly because it will be much easier for one to harvest *Ganoderma* in the wild once there is information of the hosts on which they naturally grow. Lastly, the study of genetic diversity of *Ganoderma* species in Namibia will lead to better understanding of this medicinally important genus, to conservation of *Ganoderma* gene plasma and this might lead to utilization of indigenous *Ganoderma* mushrooms in Namibia.

1.2 Objectives of the study

1.2.1 Main objectives

The main objective of this study was to determine and compare genetic diversity between different *Ganoderma* species in the north-eastern part of Namibia, using Random Amplified Microsatellites (RAMS).

1.2.2 Specific objectives

1. To identify the medicinal uses of *Ganoderma* mushrooms by indigenous people in the north-eastern parts of Namibia.
2. To identify different natural hosts of different *Ganoderma* species in the north-eastern parts of Namibia.
3. To determine the genetic diversity among different *Ganoderma* species in the north-eastern part of Namibia.

1.3 Research questions

1. What are the medicinal uses of *Ganoderma* mushrooms by indigenous people in the north-eastern parts of Namibia?
2. What are natural hosts of different *Ganoderma* species in the north-eastern parts of Namibia?
3. What is the genetic diversity among different *Ganoderma* species in the north-eastern part of Namibia?

1.4 Hypothesis of the study

1.4.1 Statistical hypothesis

1. H_o: *Ganoderma* species have no medicinal uses to indigenous people in the north-eastern parts of Namibia.

   H_a: *Ganoderma* species have different medicinal uses to indigenous people in the north-eastern parts of Namibia.

2. H_o: Different *Ganoderma* species occurring in the north-eastern part of Namibia grow on the same natural host.

   H_a: Different *Ganoderma* species occurring in the north-eastern part of Namibia colonize different hosts.

3. H_o: There is no genetic diversity among different *Ganoderma* species in the north-eastern part of Namibia.

   H_a: There is a genetic diversity among different *Ganoderma* species in the north-eastern part of Namibia.

1.4.2 Research hypotheses

1. *Ganoderma* species are used for different medicinal purposes in different communities in the north-eastern parts of Namibia, because the local uses depend on the traits of *Ganoderma* that are available in a particular community.
2. *Ganoderma* species grow on various hosts in the north-eastern part of Namibia due to genetic variation within the genus.

3. There will be genetic diversity among *Ganoderma* species due to varying evolutionary lineages and because they are generally an out-crossing species.

1.5 Relevance of the study

It is noted that, the limited knowledge and unrealised wealth of Africa's rich biodiversity by African themselves (Mshigeni, 2001a; Mshigeni. 2001b), had led to African countries spending a lot of money on products that they can produce locally. Mshigeni (2001a) further indicated that mushroom *Ganoderma* is one of the neglected rich biodiversity of Africa.

Mshigeni (2001b) clearly stated that there is a need to carry out research on *Ganoderma* species in Africa in order to exploit the medicinal potential of these mushrooms and their utilization. *Ganoderma* is believed to have the most promising resources promoting rapid socio-economic development in Africa (Mshigeni, 2001b). *Ganoderma* mushrooms are sources of essential amino acids, have low levels of cholesterol as well as high levels of unsaturated fatty acids (Mshigeni, 2001a). Lindesquist (1995) cited in Mshigeni and Chang (2001) further referred to *Ganoderma lucidum* as a "health tonic" which provides anticancer, antiviral properties, blood pressure regulation as well as enhancers of the body immune response systems.

The results of this study will lead to better understanding of *Ganoderma* species which will in turn lead to the beginning of local utilization of medicinally important

indigenous *Ganoderma* species and also lay a foundation for molecular systematics of other important mushrooms in Namibia. In addition, this is the first time the analysis of the genetic diversity of *Ganoderma* species will be conducted in the north-eastern part of Namibia. Analysis of genetic diversity between species within this genus will also help in the classification of *Ganoderma* species which is currently not clearly defined.

The north-eastern part of Namibia (Kavango and Caprivi regions) falls within the woodland biome of Namibia. It is described by ferralic arenosols (Mfune, 2005), which is dominated by 70% sand soils and a little portion of silt and clay clay fractions. These types of soils are known to be rich in iron oxides and aluminium (Singer and Donald, 2002; Foth, 1990) and are usually well drained (Singer and Donald, 2002; Foth, 1990). The north-eastern part of Namibia receives the highest rainfall ranging between 450mm to 700mm per annum and temperature ranging from 6^0C to 36^0C (Mfune, 2005). This area was chosen because of its vegetation type which is one of the favourable conditions for *Ganoderma* growth (Sripuan, Tongkao, Yamamoto, and Kumagai, 2005). It is dominated by broad-leaved deciduous trees and shrubs such as; Zambezi teak (*Baikiaea plurijuga*), mopane (*Colophospermum mpane*), wild seringa (*Burkea Africana*) and several *Acacia* species (Mfune, 2005). This area is dominated by hardwoods which are one of the *Ganoderma* species host.

1.6 Limitation of the study

Ganoderma samples could only be collected immediately after the rain season. For this reason, multiple collectors were involved to ensure adequate samples collection during the available shortest time. Different *Ganoderma* species could not be identified morphologically in the field, this led to the collection of all encountered species regardless of whether they were different individuals that belong to the same species or not.

Miller et al. (1999) stated that there are still complications when it comes to identifying different species within this genus. These are due to close similarities between different species and huge variations within species. Miller et al. (1999) continued reporting that, these complications resulted from different environmental factors in different geographical areas, heterogeneic forms and the use various unclearly defined characters during identification. Since there are still problems and confusion when it comes to identification of different species, this caused much difficulty in determining genetic variation between species because there are no distinct boundaries between species.

CHAPTER 2

2. Literature Review

2.1 Introduction

The colonisation of *Ganoderma* species on trees is noticed by the appearance of their cap shaped, hard, crusty and shiny varnished or dusted with spores' appearance of the upper surface of their fruiting bodies (Schwarze and Ferner, 2003). Fruiting bodies of *Ganoderma* are associated with very diverse morphology. This diverse morphology is further elaborated by McMeekin (2004) that it varies from the presence or absence of the stem, different colour of the outer surface of the cap, presence or absence of branches as well as different cap sizes. In particular the cap sizes seems to be determined by both genetics and environmental factors, while branching of the fruiting bodies is known to be caused by lack of light as it is normally associated with isolates collected in caves (McMeekin, 2004). Alternatively, McMeekin (2004) could not explain the cause of variation in some external features, but highlighted that there is a possibility of high genetic variability within the genus which might be the cause of great variation in morphological features observed in *Ganoderma* species.

According to Mizuno et al. (1995) cited in Buchanan (2001) *Ganoderma* mushrooms are important as a source of medicinal and neutriceutical products. Buchanan (2001) further indicated that *Ganoderma* mushrooms are also plant pathogens and they also play a major role in the decomposition of dead wood thus contributing a lot to

nutrient cycling. All *Ganoderma* species cause white rot on woody materials after colonisation and are proved to cause degradation of woody cell walls (Schwarze et al 1995). Degradation is caused either through simultaneous rot or through selective delignification. As the fungus continues to decay the host, the wood becomes progressively softer resulting in loss of stiffness of the woody materials in the latter stage of the fungus life cycle (Schwarze and Ferner, 2003).

Many authors refer to *Ganoderma* species as wood rotting fungi or a cause of decay in a very broad range of tree species throughout the world (Flood et al., 2000) and the disease caused by *Ganoderma* species as a *Ganoderma* root rot or white rot. Paterson (2006) described *Ganoderma* as a strange macro fungus that completely digests lignin hardwoods to water and carbon dioxide leaving the white cellulose exposed and available as nutrient for the fungus. The latter resulted in its common name the "white rot" fungus. Many *Ganoderma* species are saprotrophic fungi, however some species like *G. applanatum* initially colonise its hosts as a parasite and then develop saprotrophically in later stages (Petersen, 1938 cited in Schwarze and Ferner, 2003). Saprotrophs are defined by Mader (2004) as organisms which excrete digestive enzymes and absorb the resultant nutrients back through the plasma membrane. The latter will then lead to the death of many *Ganoderma* hosts which include deciduous, coniferous and other hardwood trees.

Campbell and Reece (2005) stated that fungi of phylum Basidiomycota are highly important decomposers of the wood and other plant materials. Campbell and Reece (2005) further indicated that beside all fungi basidiomycetes are the best in

decomposing the lignin polymer in woods. In addition to this Hseu et al. (1996) specifically pointed out that *Ganoderma* species are the fall within a group of microorganisms that appears to be effective in biological degradation of lignin. *Ganoderma* fungi prefer colonising old or aging trees, declining trees as well as dead woods and stumps (Hseu et al., 1996). According to Campbell and Reece (2005) basidiomycetes colonize and break down tissues of weak and damaged trees and continue to decompose further even after the tree dies. Hseu et al. (1996) further added that the type of *Ganoderma* species as well as the tree host species tends to be the two main characters which determine the types and the rate of decay caused by *Ganoderma* species on wood hosts. In fact *Ganoderma* species play a major ecological role in woodland and forest ecosystems through decomposition of trees.

Furthermore Schwarze and Ferner (2003) reported that the presence of *Ganoderma* can easily be detected through the presence of their perennial fruiting bodies which made it easy for distribution assessment of this macrofungi. Beside this, there are shortcomings caused by species such as *G. resinaceum* which reproduce annually. Consequently such species can easily be overlooked during distribution assessment.

Taxonomic divisions within the genus *Ganoderma* are very confusing. This is because of the presence of the heterogeneic forms, taxonomic obstacles (Mueller et al., 2007) and inconsistencies in application of many criteria by which the genus has been subdivided, hence all these resulted in doubtful nomenclature (Miller et al., 1999). Similarly, Sun et al. (2006) noted that this confusing situation is mainly a result of different authors using various criteria during identification. Some authors

only focus on criteria such as host specificity, geographical distribution and macro morphology of basidiomes, while some authors strictly only focus on spore characters as the primarily taxonomic characters.

Sun et al. (2006) further stated that the use of spores is very complicated and not well defined, therefore it is one of the characters that has delayed clear taxonomic ranking within genus *Ganoderma.* Accordingly in most *Ganoderma* study cases species level identification is often not attempted. Conversely, Utumo et al. (2005) noted that taxonomic obstacles within genus *Ganoderma* have been caused by a rareness of trained mycologists, lack of long term studies, and few published articles in the *Ganoderma* field.

In order to reduce this taxonomic chaos researchers are currently focusing on the use of molecular methods to identify *Ganoderma* species. Molecular methods are designed to detect naturally occurring polymorphism at Deoxyribonucleic Acid (DNA) levels (Sun et al., 2006). Molecular markers have many advantages over morphological markers, such as they are not influenced by effects of different environmental factors, physiological stage of an individual and they are not tissue specific (Daud et al., 2007; Zhou et al., 2007). For these reasons they can be detected during any developmental stage of an organism and they have high polymorphism (Daud et al., 2007; Zhou et al., 2007). Most importantly in molecular analysis is that a very small amount of the sample is sufficient for analysis and also the fact that the physical forms of samples do not restrict DNA detection.

In comparison, morphological characteristic of *Ganoderma* are known to vary with changes in environmental factors and therefore not reliable to be used as the sole principle for identifying different species (Zakariah et al., 2005). In addition, the accessibility of fungi DNA sequences in genebanks has eased the way applied molecular mycology can be practised. Furthermore, the capability to be able to identify a certain fungi by only using DNA sequences has proved the effectiveness of molecular mycology in issues where traditional taxonomic methods failed to produce conclusive stable classification groups (Bridge, 2002; Hseu et al., 1996). Further, applied molecular mycology also indicates the obscurity of using morphological methods in characterising individual fungal strains (Bridge, 2002). For these reasons molecular methods form important tools in mycology studies, hence they were opted for in this study.

To date different molecular methods are used to determine if the morphologically defined groups are supported by molecular evidence (Ferrer et al., 2000). Many researchers have made trials to identify *Ganoderma* species using isoenzymatic studies as well as morphological data. Nevertheless there are still no identification keys for all known species of the genus *Ganoderma* (Gottlieb and Wright, 1999) with exception to the most popular *G. lucidum*.

2.2 Molecular markers

Molecular markers refer to specific segments of DNA that are at specific location. They can be identified within the whole genome and they are used to identify genetic

varieties between individuals (Daud et al., 2007). In simplest form molecular markers can be defined as a mark along the DNA track that identify the location of a desirable genetic trait or that distinguish specific genetic differences. There are many different molecular methods for measuring genetic diversity. Some of these methods are non Polymerase Chain Reaction (PCR), while some are PCR based methods. There are two common non-PCR methods, namely DNA fingerprinting and the Restriction Fragment Length Polymorphism (RFLP). The PCR based methods are as follows: Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellites, DNA sequencing, Single Nucleotide Polymorphism (SNP), Single Strand Confirmational Polymorphism (SSCP) (Nepolo et al., 2009) and Sequence Related Amplified Polymorphism (SRAP) (Sun et al., 2006).

Different areas of study can be addressed by different molecular approach. For instance AFLP is regularly used to resolve individuals or groups of isolates inside a sub specific group (see Table 1). During the selection of appropriate method to be used the following factors should be considered. These are the type of sample that is available, the levels of systematics that is being investigated, cost of the process, ease of the technique, development time, inheritance pattern and the source of DNA sample (Bridge, 2002). Of all the PCR methods Zhou et al. (2007) accentuates that AFLP, SSR, RAPD and DNA sequencing require a relative quality DNA from a large population. Therefore researchers should carefully decide on the appropriate DNA extraction method or kit before using these methods.

The SRAP technique was used in previous studies to determine genetic diversity between different *Ganoderma* species (Sun et al., 2006). According to Sun et al. (2006) the result obtained in this study indicated little similarity between some strains of *Ganoderma* which means there is high genetic variability between some species of *Ganoderma*. However, the study further indicated that *G. lucidum* from Yugoslavia formed a different cluster from *G. lucidum* from China (Sun et al., 2006). This reveals that there is also genetic diversity within species. Higher genetic diversity might have been caused by different evolutionary path the population has followed over a long period of time in their region of distribution. Because of greater variation within species, many researchers have concluded that species like *G. lucidum* are different but related species due to similar fruit body color (Lin, 2001; Sun et al., 2006).

Table 1 shows different features of commonly used molecular markers in the mycology field, the DNA type that would be studied as well as the taxonomic level that would be studied by using each of the markers and lastly, the type, quality and quantity of the required sample when using any of the markers.

Table 1. Features of commonly used molecular markers (adapted from Bridge, 2002)

Marker	**DNA type studied**	**Taxonomic level resolved**	**Sample required (µg)**
RAPDs	Variable, generally nuclear, sometime repetitive	Individuals, sub-specific groups	Purified high quality DNA (0.02)
Simple repetitive PCR sequences SSR	Variable, generally nuclear, sometime repetitive	Individuals, sub-specific groups	Purified moderate quality DNA (0.05)
AFLPs	Subset of total genome	Individuals, sub-specific groups, some closely related species	Purified moderate quality DNA (0.5-1)
Mitochondrial DNA RFLP	Mitochondrial DNA	Individuals, sub-specific groups, some closely related species	Purified high quality DNA (0.02)
ITS/IGS region RFLP	Nuclear rDNA variable spacers	Some sub-specific groups, closely related species	Organism
ITS region Sequencing	Nuclear rDNA variable spacers	Some sub-specific groups, closely related species	Organism
rRNA gene sequences	Nuclear rDNA	Species, genera, families, phyla	Organism
Protein genes	Conserved coding regions	Species, genera, families, phyla	Organism

SRAP is a very useful tool in molecular marker technique, it helps in identifying same species which were given different names and also identify duplicates in

original collection example in the case of *G. lucidum* (Sun et al., 2006). Previous studies indicated that, markers such as allozymes and RFLP have been successfully used in genetic diversity analysis. However the two methods have several shortcomings including low-intra and inter-specific polymorphism (Liu et al., 2000). Thus conclusion has been made that these markers are less sufficient when analysing genotype of huge germplasm collections (Liu et al., 2000; Daud et al., 2007).

RAPD is one of the PCR based technique which is largely used in characterization of plant pathogenic fungi such as *Ganoderma* (Williams et al., 1990) cited in (Zakariah et al., 2005). Beside *Ganoderma* RAPD has also been used in studies of other plant pathogenic fungi such as *Colletotrichum fragariae*, *Aphanomyces euteiches* and *Fusarium moniliforme* (Zakariah et al., 2005). Hseu et al. (1996) acknowledges that RAPD is one of the useful tool for systematics at the low taxonomic level especially in cases where isolates have similar ITS sequences and hence can not be resolved by using ITS data.

2.2.1 Microsatellites (SSR)

Microsatellites refer to a region within DNA sequence (loci) where short sequence of DNA nucleotide (adenine (A), guanine (G), thiamine (T) and cytosine (C)) are repeated in tandem arrays (Tan et al., 2006). This simply means the sequences are repeated one after another for example CACACACA, and they tend to be found in non-coding DNA. The sequences may vary in number of repeats; they can either be di, tri, tetra or more. Some literature refers to microsatellites as Simple Sequence

Repeats (SSR), Short Tandem Repeats (STR) or Variable Number Tandem Repeats (VNTR).

Microsatellites are very useful due to the fact that the number of repeats of a particular sequence varies between individuals, populations, between species and they are known to be the most advanced marker technologies (Tan et al., 2006). However, the repeats occur at the same location on the genome. This characteristic plays a major role during the identification of different species. Depending on the rate of change, areas that have high rate of mutation are associated with a diverse range of number of repeats within individuals in a population. In addition, the usefulness of microsatellites varies in different studies depending on the variability of a particular microsatellite. For example, microsatellites associated with few varieties as well as low level of mutations, have a crucial role in analysis of related species. If the microsatellite has a high variability and there is a large number of alleles shared between population species then it is less useful in identification. This is because it results in uncertainty as to which species does an individual belongs to.

Microsatellites enable the differentiation of genetic diversity between different populations. Microsatellites are valuable markers for identification of relationships and genetic diversity (Zhang et al., 2004; Wendel and Cronn, 2003). This is due to uniform distribution, high polymorphism, high abundance, rapid PCR amplification, ease of use, co-dominant inheritance (exhibit Mendelian inheritance), multi-allelism, hypervariability (Tan et al., 2006), easy interpretation and high availability of SSRs to researchers world wide through published primers sequence (Saghai-Maroof et al.,

1994; Daud et al., 2000). Furthermore, Bal and Akkaya (2002) considered microsatellites as one of the most dominant molecular markers for various diverse applications in genome associated studies. Lastly microsatellites are more useful in fungi genetic studies because they are present in higher abundance throughout eukaryotic genome (Tautz and Renz, 1989) and also because they evolve rapidly (Levinson and Gutman, 1987).

Random Amplified Microsatellites (RAMS) is another PCR based molecular marker which can be used in genetic diversity. RAMS are made up of a combination of both RAPD and Microsatellites characteristics (Zakariah et al., 2005). It is clearly stated by in Zakariah et al. (2005) that Hantula et al. (1996) had proven that RAMS is applicable and reliable to be used in studies of genetic variation of fungi.

2.3 PCR amplification

Polymerase Chain Reaction (PCR) is one of the commonly used techniques in molecular biology. This technique was first described in the mid 1980s. PCR is defined by Bridge (2002) as the procedure by which multiple copies can be made out of a certain DNA piece. This process aims at amplifying a targeted tiny DNA region of a particular sample. PCR is described by (Manzanares-Dauleux et al., 2001) as a potential molecular method for assessing genetic variation. Shaw et al. (1999) added that DNA based markers have a high power of discrimination that enable them to differentiate even closely related species. Bridge (2002) further explain that PCR works by firstly converting the targeted double stranded DNA to single stranded

DNA, followed by the attachment of primers to each end of the region of interest. The PCR reaction makes use of an enzyme taq polymerase to multiply DNA molecules in a PCR tube up to a thousand folds (Madigan and Martinko, 2006).

Taq polymerase will construct the area between the primers from the targeted DNA by adding individual phosphorylated nucleotides (Brigde, 2002). This will yield a large amount of specific genes from the fungus sample. The primary condition of PCR is temperature variations. The reaction requires very high temperature of above 90^{o}C to denature double stranded DNA as well as low temperatue of 34^{o}C-55^{o}C for primers to bind to the targeted DNA region (Bridge, 2002). Taq polymerase remains stable up to 95^{o}C and therefore does not get denatured by high temperature. According to Saiki et al. (1988) cited in Bridge (2002) the complement goes to the discovery of heat resistant enzymes in the thermophilic bacteria *Thermus aquaticus* that led to the use of taq polymerase that made PCR to become a viably practical process. Because of this the PCR can be run at range of 35^{o}C-94^{o}C. According to Madigan and Martinko (2006), the use of polymerase at a high temperature is the one that results in more homogenous products during DNA amplification.

During DNA amplification, DNA oligonucleotide forward and reverse primers will be added to a heat denatured target DNA. As the mixture cools down the primers that were added to the DNA in excess will ensure that most of the target DNA has annealed to the primers other than annealing to their complementary strands. After this, DNA polymerase will begin to extend the primers with the target strand

templates. The last step takes place at Taq polymerase optimum temperature which allows the formation of new DNA.

It should be noted that PCR is only a process of acquiring large amount of targeted DNA, but it is not an analysis. Therefore it is crucial to analyse the features of the amplified DNA. The end product of PCR varies depending on the method used and kind of primer used. For example in diagnostic studies where specific primers are used, the end product may be either the presence/absence of the targeted DNA in a sample or it can be the size and the number of product in a case where generalised primers are used (Bridge, 2002). PCR is an extremely sensitive method in such a way that it can detect tiny pieces of DNA. On the other hand the sensitivity of the PCR amplification depends largely on the specificity of the primers. In the simplest form that is the ability of the primer to detect targeted DNA sequences when they are present in a complex DNA mixture.

2.4 Influence of reproduction on genetic diversity

The main unit of diversity of life is DNA (Campbell and Reece, 2005). This is a unity of inheritance which is passed on from parents to offspring through reproduction. Basidiomycete fungi reproduce sexually (Webster and Weber, 2007). During sexual reproduction there is reshuffling of genetic materials during meiosis. As a result, usually sexually reproducing organisms are associated with higher genetic diversity than asexually reproducing organisms. See the life cycle Basidiomycete in Figure 1. The basidiomycetes lifecycle has three important

phrases: the haploid phase (n), dikaryotic (n + n) and the diploid phase (2n), which are formed through three different process karyogamy, meiosis and plasmogamy, see figure 1 (Webster and Weber, 2007). Dikaryotization is defined by Webster and weber (2007) as a procces by which two hyphal walls rupture causing two cytoplasma to combine (plasmogamy) without the fusion of the nuclei from the two combining cells. The mycelium that develops from this cytoplasmic continuity is called dikaryotic. According to Webster and Weber (2007) this process only takes place in the Kingdom of Fungi however it does not apply to all species.

The most important feature of the basidiomycetes is the presence of dikaryotic. Dikaryotic mycelium responds to environmental stimuli such as rain to reproduce sexually by forming compact masses that develop into basidiocarps. Within the basidiocarp gills are terminal dikaryotic cells called basidia (see Figure 1). Each dikaryotic cell in the basidia undergoes karyogamy to produce 2n cells that will further undergo meiosis resulting into four haploid nuclei. Karyogamy is also another crucial process for source of genetic variation in somatic diploid cells (Wester and Weber, 2007). It is a process by which pronuclei of two cells fuse. This process takes place before meiosis. Campbell and Reece (2005) stated that during prophase 1 of meiosis genetic rearrangement between non sister chromosomes occurs.

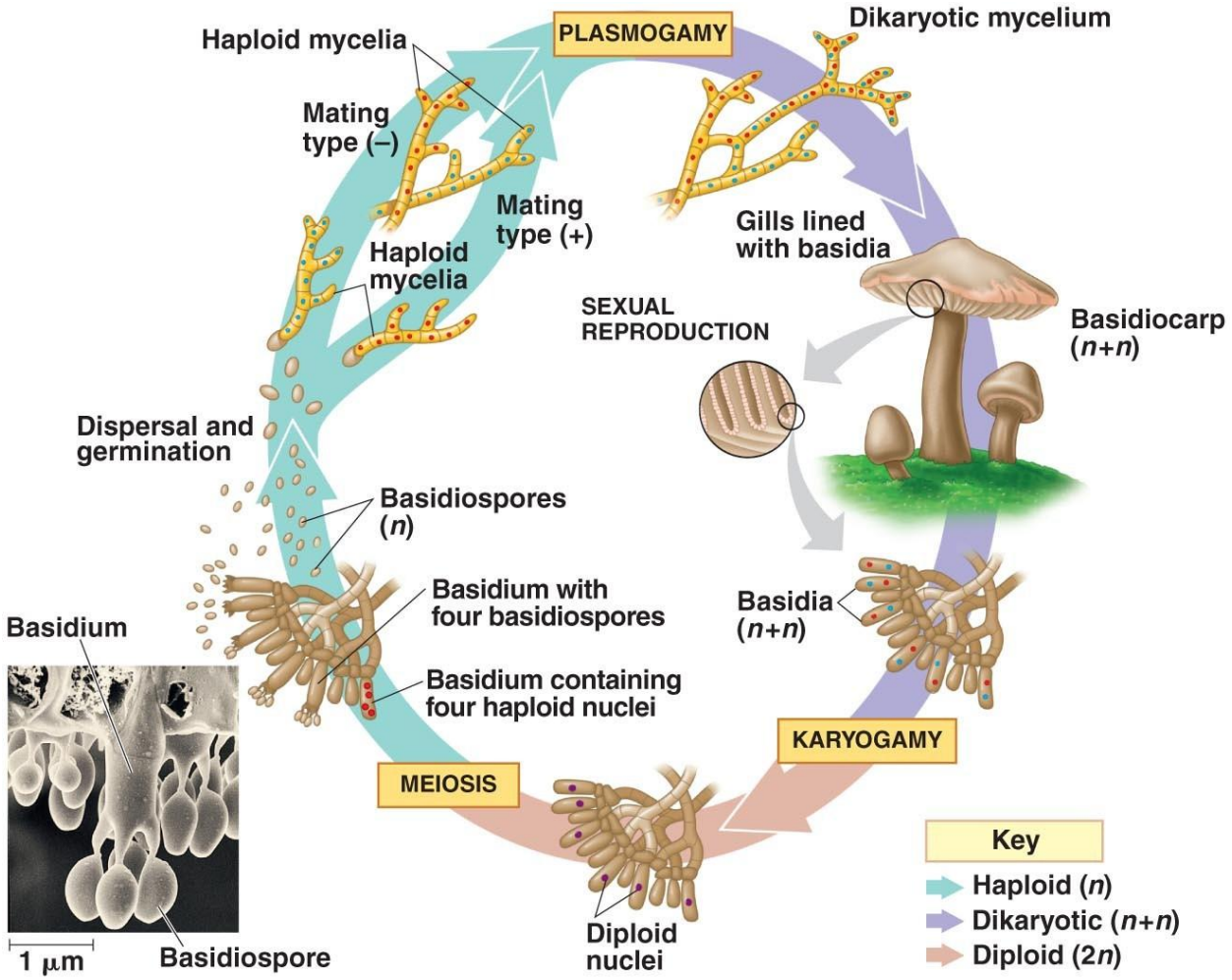

Figure 1. A typical life cycle of the mushroom-formimg Basidiomycete Adapted from Campbell and Reece (2005, p. 619)

Campbell and Reece (2005) further elaborated that in a process called crossing over of prophase 1 the DNA molecules in non sister chromatids break at corresponding places and then re-join the other chromatid's DNA. Beside crossing over, the other two processes responsible for genetic variation during sexual reproduction are independent assortment of chromosomes of meiosis and random fertilization (Campbell and Reece, 2005). Therefore it is this random orientation and recombination of chromatids and homologous pairs which occurs in meiosis that generates great genetic variation in generation of sexually reproducing organism.

Each haploid nucleus grows into one appendage on the edge of basidia (Figure 1). These appendages grow into basidiopores which will be dispersed by wind after they mature. Under suitable environmental conditions a basidiospore germinates into short-lived haploid mycelia (Campbell and Reece, 2005). Two haploid mycelia mate to form a long-lived dikaryotic cell which will then grow and mature to form fruiting bodies.

CHAPTER 3

3. Materials and Methods

3.1 Research design

The samples were collected from north-eastern part of Namibia which was divided into two regions Kavango and Caprivi region (Figure 2). Field work was conducted between March 2010 and May, 2010 for collection of field samples from the Kavango and Caprivi region. This field trip specifically focused on the collection of all mushrooms species that belong to the genus *Ganoderma.* For all encountered individuals, a field sample was collected and necessary data was recorded for analysis in the laboratory.

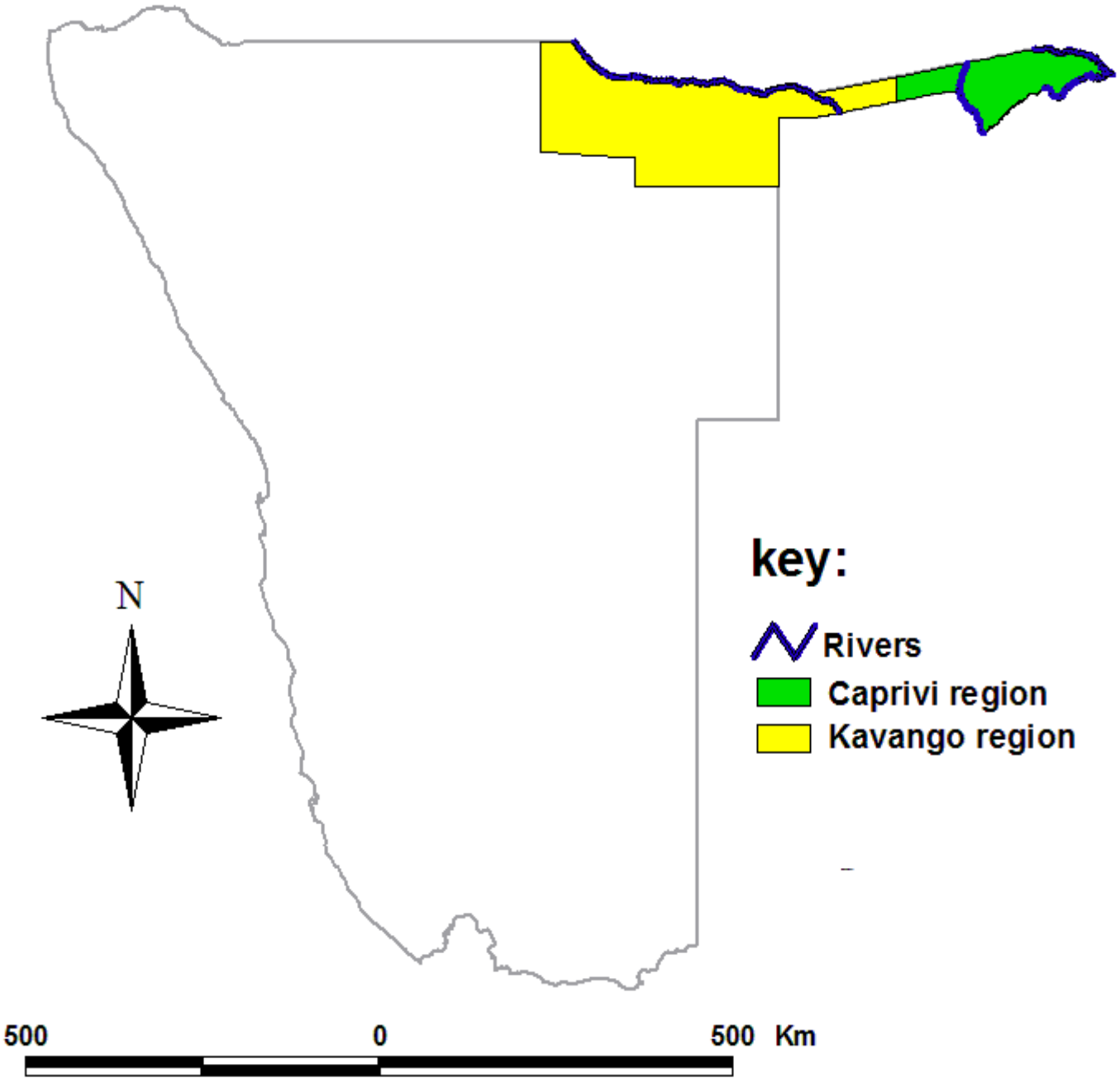

Figure 2. Two regions in the north-eastern part of Namibia where *Ganoderma* samples were collected for this study.

All samples collected in the field were brought to the molecular biology laboratory at the University of Namibia for DNA extraction. After collection of samples from the field, several steps that lead to capturing of PCR data were carried out in the laboratory.

In each region five individuals representing the encountered *Ganoderma* isolates were collected. All the collected samples were brought to the molecular biology laboratory at the University of Namibia for genetic diversity analysis. Lastly RAMS were used to determine genetic variation between different species of the genus *Ganoderma*.

3.2 Population

This research focused on various populations of all different *Ganoderma* species. All populations that were encountered during the field work in the north-eastern part of Namibia were considered. A target of at least three isolates per subpopulation was aimed at.

3.3 Sample

This research was conducted at molecular level. Hence it only aimed at obtaining adequate sample sizes for molecular analysis. In this study, 3 individuals were collected from each encountered sub population.

3.4 Research instruments

Collected *Ganoderma* samples were stored in khaki paper bags. *Ganoderma* tissues were grinded to powder using mortar and pestles. DNA was extracted from 200 mg *Ganoderma* powder. Other instruments that were used in this research include a Cycler PCR machine by Bio-rad that was used for DNA amplification, agarose gel trays and an electrophoresis machine which was used to run microsatellite products. Lastly, other important instruments that were used were eppendorf tubes that were used for storing extracted DNA samples and centrifuge machines.

3.5 Procedure

3.5.1 *Ganoderma* samples collection

In both regions, areas with dense and highly stratified woody vegetation were targeted for the *Ganoderma* search. In addition to this, cultivated fields that were densely populated with dead stumps and woody trees were also considered. During field work the field guide "Mushrooms of Southern Africa" by Westhuizen and Eicker (1994) was used to differentiate *Ganoderma* species from other similar polyporaceae fungi such as *Pycnoporus sanguine* (see Figure 3).

All collected samples where dried in a well ventilated room for two weeks prior to DNA extraction. After drying the samples were then stored in dry clean room. During sample collection GPS points (Appendix 3) were stored in the GPS and later

uploaded on Garmin Map source software eTrex HC/HCx at the end of the survey to mark sampled sites.

Figure 3. *G. ludicum* (A) and *Pycnoporus sanguineus* (B)

In addition, other necessary information such as the host identity dates on which the samples were collected and other necessary biodata that might have led to the characterization were also collected. Last of all pictures of *Ganoderma* fruiting bodies on their natural hosts were also taken in the field.

3.5.2 *Ganoderma* uses Questionnaires

A questionnaire is one of the best ways to collect qualitative data, most specifically when the information is not documented. A questionnaire is also one of the best ways to study human knowledge or other characters that cannot be observed. Indigenous knowledge can be defined as the local knowledge that is unique to a given culture or society (Mapaure, 2008). Mapaure (2008) and Matowanyika, Garibaldi and Msimwa (1994) further indicated that indigenous knowledge is not documented and is mainly found in old individuals in the society. To gather information on the traditional medicinal uses of *Ganoderma* mushrooms by indigenous people in the Kavango and Caprivi regions, an open ended format questionnaire was used. Open ended format was opted for because it can accommodate a variety of responses and more truly reflect the opinions of the respondents.

Questionnaires were used in a face to face interview to ensure the answering of all questions. The questionnaire was read out to each and every individual in their vernacular language and the answers were filled in English by the translator. This was done because the targeted group of people in this study were elders who mostly

did not have the reading skills. The findings were summarised later after the survey and are presented later in the results in a pie chart (Figure 4).

3.5.3 Hosts assessment

The host of each collected sample was identified. The "Southern Africa tree field guide" by Van Wyk and Van Wyk (1997) was used to identify *Ganoderma* hosts in the field. Tree hosts that could not be identified in the field had specimens sharply cut off sharply using secateurs and stored in a plant press. These specimens were taken to National Botanical Research of Institute (NBRI) for identification to species level. For dead stumps, villagers were requested to identify a live tree of the same species, on which good specimen could be collected for later identification. In cases where no life could be identified or no one knew what tree it was the barks of stumps were collected for later identification.

3.5.4 DNA extraction

Filamentous Fungi have rigid cell walls and high polysaccharides which pose difficulty during DNA extraction. For this reason Zhou et al. (2007) strongly advised researchers to use extraction methods that enhance breaking of cell walls. Zhou et al. (2007) further indicated that methods like CTAB, SDS-CTAB, SDS methods and benzyl chloride method are only suitable when DNA is extracted from mycelia. In this study DNA was extracted from *Ganoderma* tissues using Zymo Research (ZR) Bacterial/Fungal DNA kit. Prior to DNA extraction the *Ganoderma* was ground to powder using sterilised mortars and pestles.

Following the ZR instruction manual, DNA extraction started with addition of 200 mg of *Ganoderma* powder into ZR bashing bead lysis tube. Following this, 750 µl of the ZR lysis solution was added and the tube was vortexed at a maximum speed for five minutes using a standard bench vortex. The ZR bashing bead lysis tube with *Ganoderma* mixture was centrifuged in a microcentrifuge at 10 000 rpm for one minute. A volume of 400 µl of the supernatant in the bashing bead lysis tube was transferred into a Zymo spin IV spin filter placed in a collection tube and centrifuged for 1 minute at 7000 rpm. This was followed by addition of 1200 µl of the bacterial DNA binding buffer to the filtrate in the collection tube from the previous step. An 800 µl portion of the mixture in the collection tube was transferred into a Zymo spin IIC column in a collection tube and centrifuged at 10000 rpm for one minute.

The flow through from this step was discarded and the step was repeated using 800 µl of the mixture that was left in the collection tube. The Zymo spin IIC column used in the previous step was transferred in a new collection tube and 200 µl of bacterial DNA pre-wash was added to the IIC column followed by centrifuge at 10000 rpm for one minute. After centrifuging 500 µl of fungal / bacterial DNA wash was added to the same IIC column and the tube was centrifuged for one minute at 10000 rpm. The IIC column from the previous step was placed in a sterilised eppendorf tube. There-after, the extracted DNA was eluted by pipetting 100 µl of DNA elution solution directly on the IIC column matrix and centrifuging the IIC tube at 10000 rpm for 30 seconds. The extracted DNA solution was prepared for gel electrophoresis by mixing 20 µl of the extracted DNA with 4 µl of Promega loading dye in a new sterilised micro centrifuge tube. Finally, the solution was analysed for

the presence or absence of DNA by loading 10ul on 1.5 % Agarose gel stained with 3 ul of Ethidium Bromide. This gel was run using 0.5 % Tris Borate EDTA (TBE) buffer, in order to determine if the extracted solution contain DNA.

3.5.5 RAMS PCR amplification

Polymerase Chain Reaction (PCR) was used for DNA amplification using three different RAMS primers adapted from Hantula et al. (1996). These primers were ACA; CCA and CGA. Hantula et al. (1996) further indicated the sequence of each primer as follow: 5` BDB $(ACA)_5$, 5`DD$(CCA)_5$ and 5'DHB$(CGA)_5$; whereby H, B, Y and D where used for degenerate sites. In this regard the degenerate sites were defined as follow H = (A,T or C); B = (G or C); Y = (G, A or C) and D = (G, A or T) (Hantula et al., 1996; Zakaria et al., 2005).

A volume of 25ul was used for PCR amplification. This contained 12.5 µl of Fermentas Dream *Taq*^{TM} Green PCR Master Mix (2x), 10.5 µl of Nuclease free water, 1 ul of microsattelites primers and 1ul of DNA template. Fermentas Dream *Taq*^{TM} Green PCR Master Mix (2x) contains *Taq* polymerase, buffer, $MgCl_2$, dNTPs and loading dye. The protocol used to run PCR was adapted from that of Hantula et al. (1996, p. 161). First step was the denaturation of DNA double strands for 4 minutes at 95^{o}C for 1 cycle adapted from the Fermentas Dream *Taq*^{TM} Green PCR Master Mix (2x) protocol. PCR amplification was repeated for 35 cycles, in which denaturation was carried out for 30 seconds, annealing depending on primer specificity for 1 minute and extension for 2 minutes. The respective annealing

temperature for each primer where as follow CCA was 64^{o}C, CGA was 61^{o}C and for ACA was 49^{o}C. The amplification processes was finished with a single final extension at 72^{o}C for 7 minutes. All PCR amplifications were done using a Bio-Rad Thermal Cycler.

Table 2. Sequences and Annealing temperatures of the primers that were used in this study (Obtained from Hantula et al., 1996)

Primers	**Sequences**	**Annealing Temperatures ^{o}C**
ACA	$(ACA)_5$	49
CGA	$(CGA)_5$	61
CCA	$(CCA)_5$	64

3.5.6 Gel electrophoresis

Fermentas Dream *Taq*^{TM} Green PCR Master Mix (2x) PCR products were loaded directly on the gel after amplification as the master mix contains loading dye. PCR amplification products were separated by electrophoresis in 2.5 % Agarose using 0.5 % Tris Borate EDTA (TBE) buffer. The gel was run for 60 minutes at constant voltage of 90V. The gel was stained with 1 µl per 100 ml of Ethidium bromide and it was viewed under UV fluorescent light then photographed using a Canon PowerShot SX 120 IS camera. Pictures of each gel were captured after 40 minutes and 60 minutes respectively. In this study 100 base pair QIAGEN Gelpilot® DNA molecular weight marker was used as a molecular size standard for evaluation of amplified DNA band sizes.

3.5.7 Data analysis

The PCR products were scored absence (0) and presence (1) for non-amplification and amplification fragments and recorded in a binary matrix as shown in Appendix 2. In this study three methods were used to analyse data obtained from amplified RAMS banding patterns. These were: an unweighted method of the UPGMA (Unweighted Pair Group Method with Arithmetic Mean): Cluster Analysis (CA) (Saitou and Nei, 1987 cited in Demey et al., 2008) and a weighted method Principal Coordinates of Analysis (PCoA) (Demey et al., 2008). The use of both ordination and cluster diagrams and comparisons of results of the same data set is recommended by Waite (2000) as similar results are expected from both methods. Beside this an old method for calculating diversity index (Shannon-Wiener index of diversity) was also used. The first two methods were also recommended in Demey et al. (2008) as appropriate and popularly used for ordination of individual genotypes using DNA marker scores. Similarity matrices were developed for rows (individuals) by columns (bands). CA was performed using group average cluster mode and the results were presented in dendrograms. CA was performed using Primer 5 version 5.2.0 statistical software (Takundwa et al., 2010).

The genetic diversity was calculated using Shannon-Wiener index of diversity by the formula below adapted from (Smith, 1990; Monaghan and Halloran, 1996):

$$H' = -\sum_{i=1}^{s} (pi)(logpi)$$

where: H = diversity index

s = number of bands

i = bands number

pi = the frequency of i^{th} band among all individuals in a population

H values from two populations were averaged to give average per locus genetic diversity within populations (H_w). Two values of H_w were further averaged to give H_{av} (average within population genetic diversity) for each primer. The average genetic diversity per population (H_d) was calculated from different H_w of three primers.

PCoA is an important tool used in individual genotypes. PCoA started with a formulation of a dissimilarity matrix from an unweighted binary matrix using Euclidean distances. This was followed by a PCoA with squared cosines of principal coordinates obtained from the proximity squared matrix formulated when 89 isolates of *Ganoderma* were compared to each other. Accordingly PCoA aim is to create a low dimensional biplot on which the distances between the points on the graph represent the original genetic dissimilarity between the individuals. By employing PCoA multiple Eigen values which define the amount of variation that is displayed on the PCoA axis as percentages of total variation were calculated (Mohammadi and Prasanna, 2003). The first two dimensions with the highest total variation percentages' were represented in a two dimensional biplot (Figure 8). According to Mohammadi and Prasanna (2003) the two axes of PCoA graph are independent of each other, each one of them reveal different properties of the data set and therefore

can be interpreted independently. All PCoA analyses were calculated using XLSTAT 2011 software (Lücking et al., 2011).

CHAPTER 4

4. Results

4.1 *Ganoderma* uses

None of the people who filled the questionnaire in Kavango region knew any use of *Ganoderma*. However they were aware of the existence of these fungi in the area and they knew where they where found. Of all the respondents from both regions, 64% did not know any use of *Ganoderma*. The most common use of *Ganoderma* was the use for hardening infant's forehead bones (infant bones) with 19%, followed by the use to halt nose bleeding (nose bleeding) with 13%. The last and less common use was to boost pregnant mother immunity (immunity) with 4% (Figure 4).

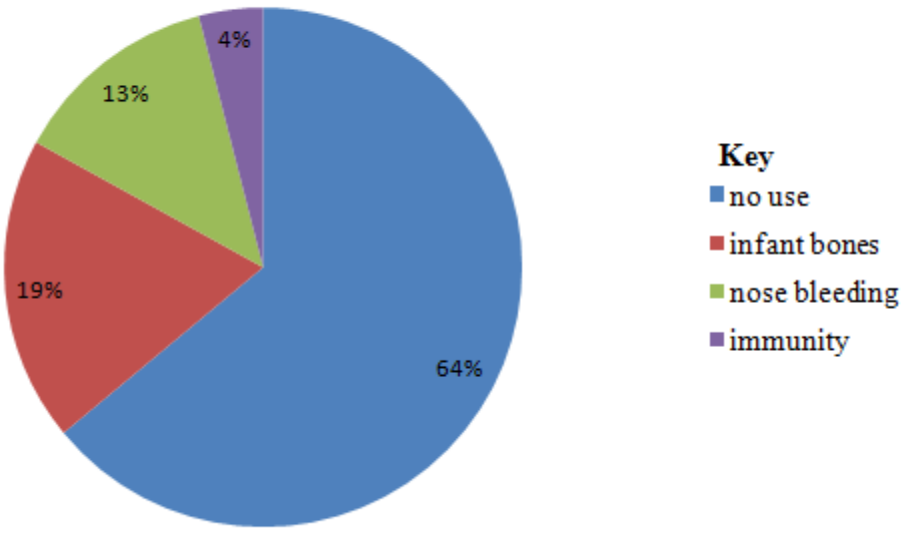

Figure 4 Medicinal uses of *Ganoderma* by indigenous people in the north-eastern parts of Namibia

4.2 *Ganoderma* Hosts

In Table 3 there are 12 different tree species that were identified as *Ganoderma* species hosts in the Kavango and Caprivi region. Table 3 also consist of the host types which vary from dead burned stumps, dead unburned stumps, live trees and debris. The same table also exhibits proportion of the samples that were collected from each host with regard to the total number of *Ganoderma* samples collected in this study. Unknown stumps accounted for 37 %. For tree hosts *Colophospermum mopane* was the one with the highest number of samples 18 % and *Ochna pulchra* and *Grewia bicolor* were the hosts with the lowest number of samples, both with 1%.

Table 3. Natural hosts of *Ganoderma* species in the north-eastern parts of Namibia

Host trees	**Host types**	**Percentages (%)**
Unkown	Dead stumps	37
Colophospermum mopane	Dead burned and unburned stupms, sprouting stumps	18
Terminalia sericea	Dead stumps, sprouting stumps, live trees	13
Combretum collinum	Dead stumps	7
Humus rich soil	Vegetation debris	4
Baikiaea plurijuga	Dead burned stumps	3
Grewia avellana	Sprouting stumps	3
Acacia erioloba	Dead stupms	3
Combretum imberbe	Dead and sprouting stumps	2

Dialium engleranum	Dead burned stumps	2
Guibourtia coleosperma	Dead burned stumps	2
Peltophorum africanum	Live trees	2
Ochna pulchra	Dead stumps	1
Grewia bicolour	Dead stumps	1

4.3 PCR Amplification

A total of 95 *Ganoderma* samples were collected. Of these six samples were discarded for various reasons as a result only 89 samples where used for PCR amplification. These reasons where mainly missing samples and moulded samples. All three primers ACA, CCA and CGA used in this studies produced amplified products for most *Ganoderma* isolates used in this study except 2k3, k6, 3m9, 2m30, 3m5, 3m20, 2m21, 2m7, 2m38 and k3 for primer ACA, k18, k13, k16, k1, 2m30, 3m9, 2m21, 2m29, 2m20, 2m32, 2m27, 2m22(b), 2m34 and k1 for CCA and 3m20 for primer CGA. A total of 43 alleles were observed from 89 *Ganoderma* isolates using RAMS.

Figure 5 below shows the amplified bands of different *Ganoderma* samples (well 2 to well 19) using primer ACA (Figure 5a), CCA (Figure 5b) and CGA (Figure 5c) in comparison to the 100 bp DNA ladder in the first well (1). Primer CGA produced many bands and most highly polymorphic bands than the bands produced by Primer CCA and ACA as it can be seen in the figure 5a, 5b and 5c below.

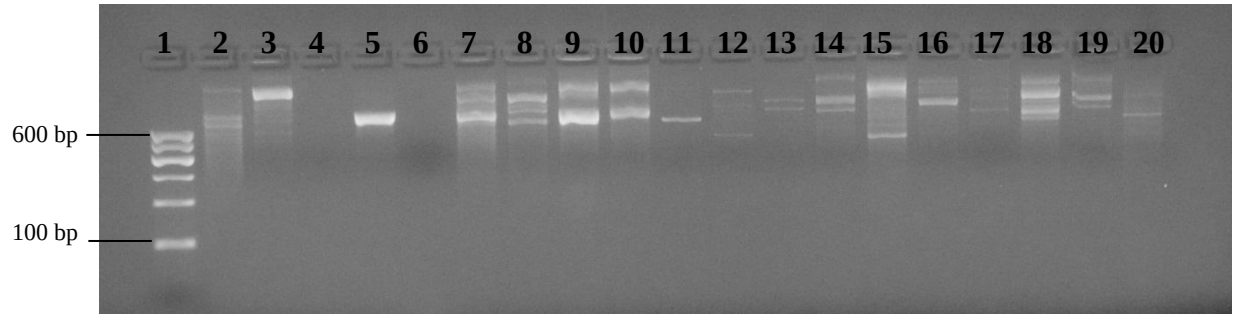

Figure 5a. PCR amplification bands of ACA primer in a 2.5 % agarose gel

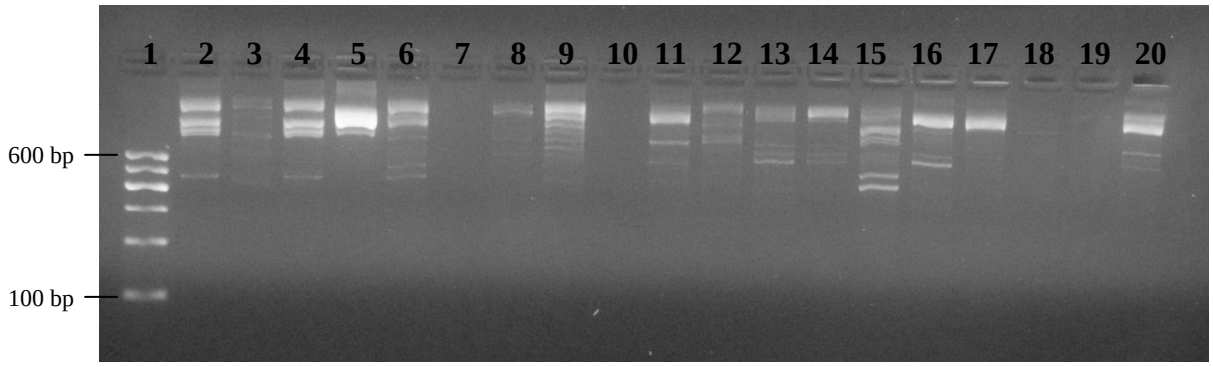

Figure 5b. Electrophoresis 2.5 % gel exhibiting PCR bands of primer CCA

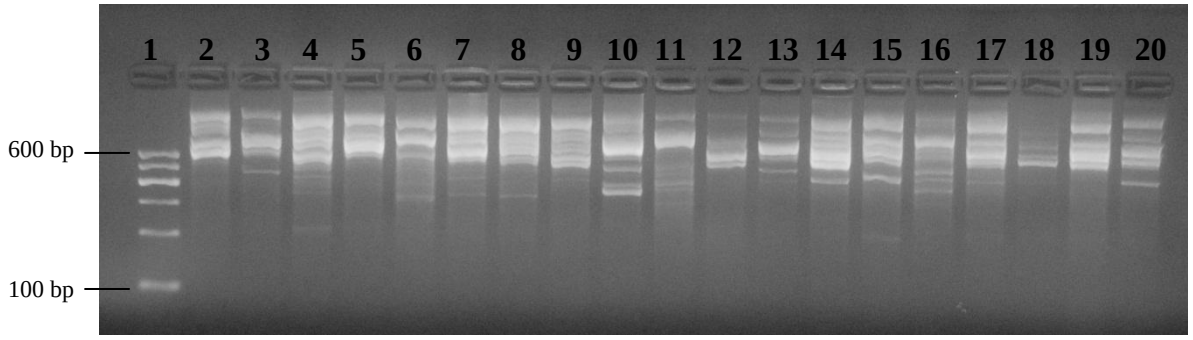

Figure 5c. Electrophoresis 2.5 % gel exhibiting PCR bands of Primer CGA

A binary matrix (Appendix 2) of amplified and non amplified PCR bands was developed from electrophoresis gels. This matrix was used for the construction of CA dendrograms (figure 6a, 6b, 6c and 7) as well as for genetic diversity analysis in Appendix 2.

Three dendrograms were constructed (one for each primer). The same samples were assessed by all three primers separately. Using Primer 5 software, the dendrogram of primer ACA ($(ACA)_5$) divided the samples into 7 clusters of different sizes at different similarity levels (Figure 6a). These seven clusters split further into multiple smaller small clusters. Cluster 3 had two samples namely 2m33 and 2m37 with 100% similarity, cluster four had various samples with 100 % similarity. These were k16, 2m17; 3m14, 2k6, 2m29, 3m18; m4; 2m35, 2m36; 2m5, k2(b), 2m1, 2m2, 2m26, 2m32; k10, k17; 2m13, 2m19, 2m22(b), 2m31, 2m4, 3m3, k1, k3; k13, k15; 3m7, 2m11, 2m28; 3m17, k12; 2m18, m1; 2m20, 2m34; k2(a), 2m12, 2m25; lastly cluster five had samples with 100 % similarity namely 2m7, 3m20, 3m5, 3m9, k6, m3, 2k3, 2m21, 2m30, 2m38; 2m22(a), 2m27; 3m4, 2m24, 3m2.

CCA ($(CCA)_5$) dendrogram firstly split into two main distinctive clusters (cluster A and cluster B) at 82 % similarity level (Figure 6b). Cluster A consisted of clearly split clusters with many samples with 100 % similarity at a genetic level (Figure 6b). Cluster B had vastly branched and squeezed clusters with very few samples that exhibit 100 % similarity. Cluster A further split at 84 %, 86.5 % and at 91 % similarity level respectively resulting into four sub clusters. On the other hand cluster B divided into three sub clusters at different significant levels. Subcluster 2 consisted of 16 samples of which fourteen indicated 100 % similarity level. These are k15, k2(a); 3m13, 2m38, 3m10; 3m7, 3m11, 3m2; 3m5, 3m6; 2m35, 2m6, 3m19, k5. In sub cluster 3 the following samples exhibited 100 % similarity level: 2m4, 2m10, 2m13; k18, m1, 2m20, 2m21, 2m22(b), 2m27, 2m29, 2m30, 2m32, 2m34, 3m9, k1, k13, k16. In sub cluster 4 it was 2m11, k14; k3, 2m19, 3m15; k8, 3m14,

k12; 2m17, 2m18, 2m31, 3m3, 3m8, k10, k6 and m2 which had 100 % similarity. Of the few samples of cluster B which exhibited 100 % similarity were 2m28 and k17 of sub cluster 5, 2m15,2m16; 2k6, 2m7 and 2m12 and 3m18 of sub cluster 6.

The dendrogram of primer CGA ($(CGA)_5$) resulted into a numerously branched CA tree which was grouped into 6 at different similarity level with very few samples with 100 % similarity (Figure 6c). This dendrogram firstly split at 77 % similarity level resulting into two cluster of which the minor cluster consisted of only two samples (2m7 and m4) while the major cluster consisted of the rest 87 samples. Of the few samples which exhibit 100 % similarity were 2k1, k17; k3,2m35, 2m5; 2m2,2m21, 2m27, m6; 2m19, m7, 2m15, 2m34; 2m13, 2m18, 3m14, m3; 2m25, 3m4, 3m5, m2; 2m20, 3m17 of cluster 2 and 2m14, k16 of cluster 6.

The overall CA dendrogram of all the three primers combined also demonstrated the existence of genetic variation in *Ganoderma* species with no *Ganoderma* isolates that exhibit 100% similarity as in the previous dendrograms of individual primers (Figure 7). The average similarity values of these RAMS widely range from 85.5% to 98.2%. The combined primers' dendrogram was divided into 10 clusters as indicated in figure 8. Even though numerous clusters were formed by each primer during the Cluster Analysis (CA) analysis, none of the dendrograms (Figure 6a, 6b, 6c and Figure 8) exhibit *Ganoderma* isolates from Kavango region clustering separately from *Ganoderma* isolates from Caprivi region.

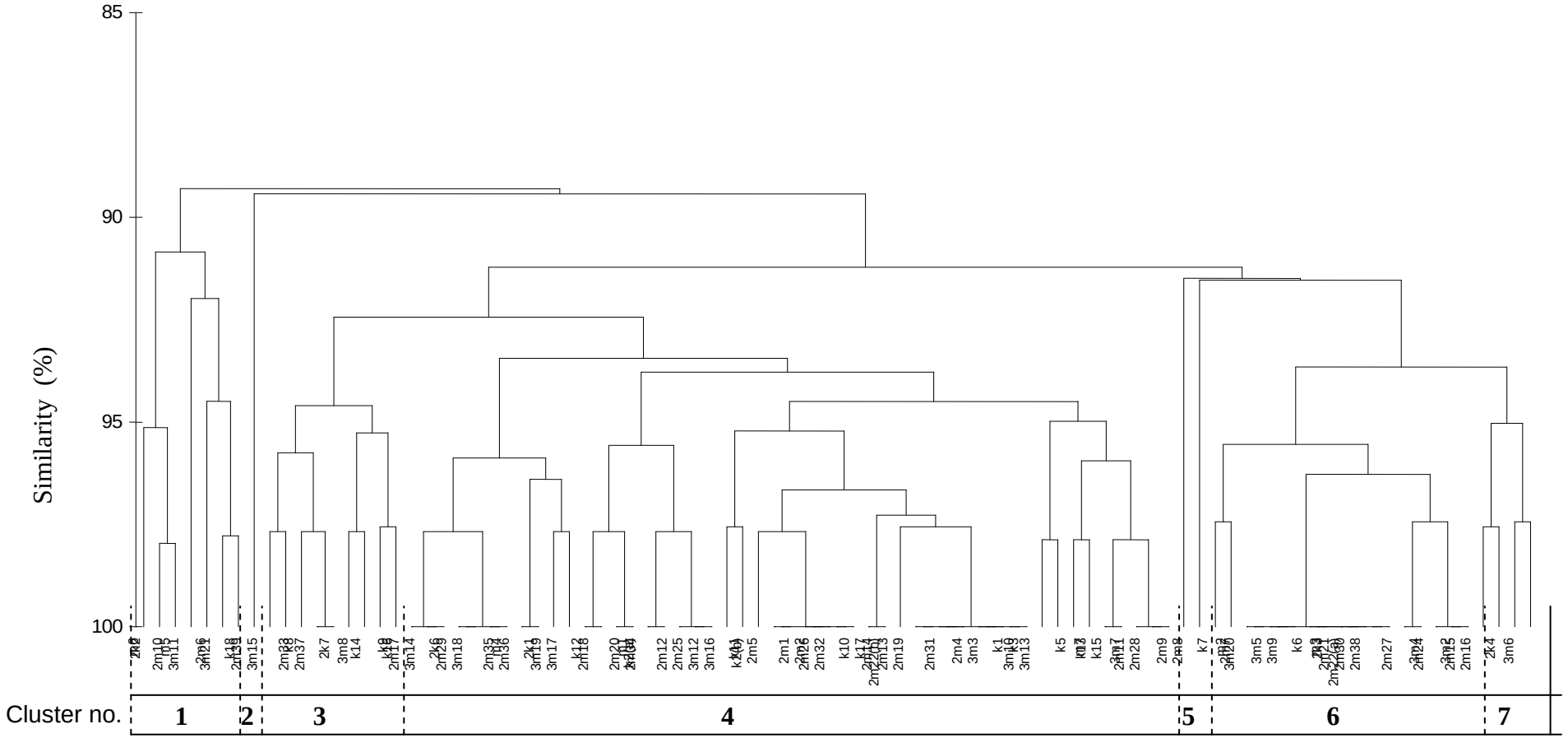

Figure 6a Cluster Analysis (CA) dendrogram showing a similarity of amplified PCR bands based on the absences/ presence of bands ACA. The letter in the labels refers to the region (e.g. M6, M= Caprivi region, 6= sample six, K= Kavango region)

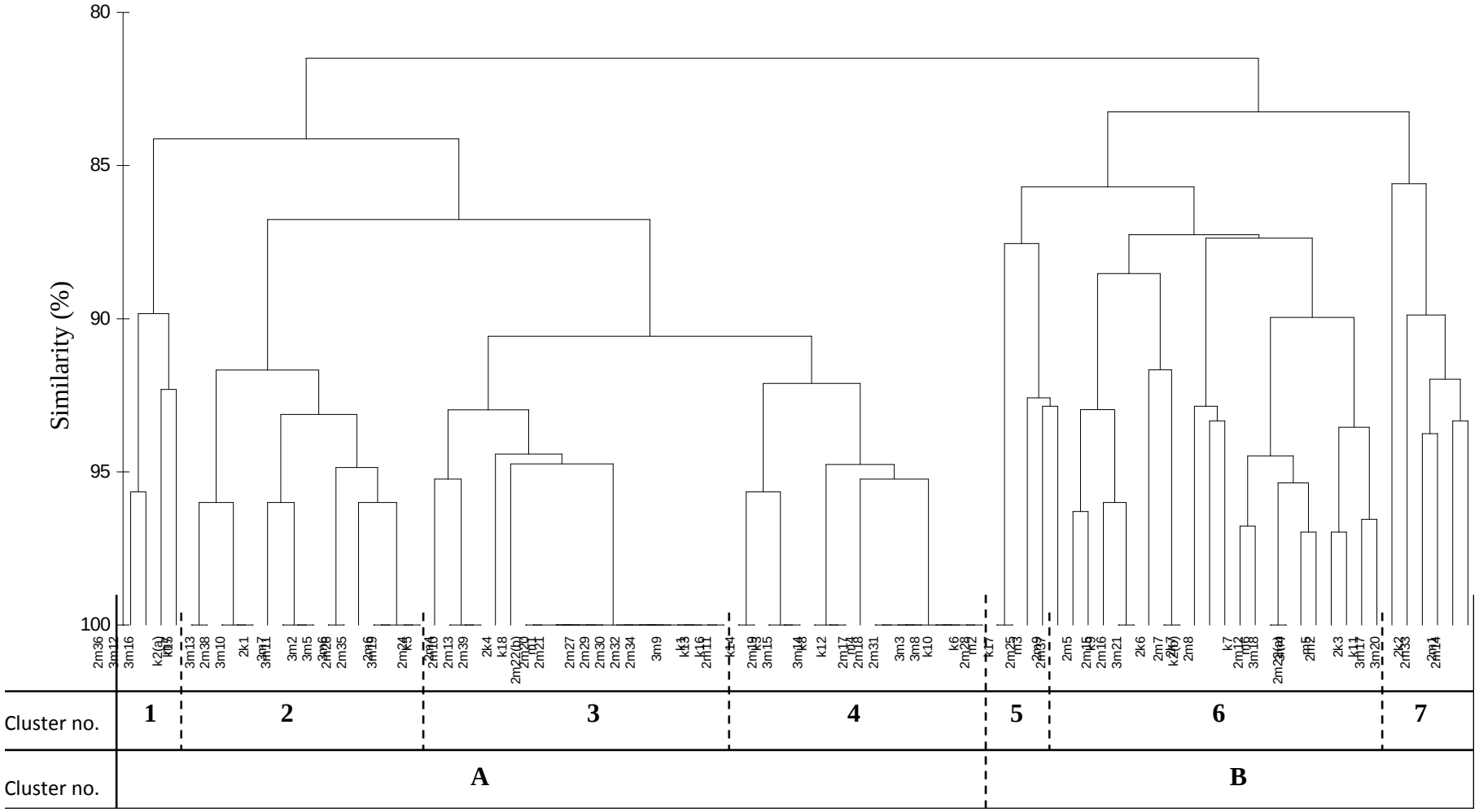

Figure 6b. Cluster Analysis (CA) dendrogram showing a similarity of amplified PCR bands based on the absences/ presence of bands CCA. The letter in the labels refers to the region (e.g. M6, M= Caprivi region, 6= sample six, K= Kavango region)

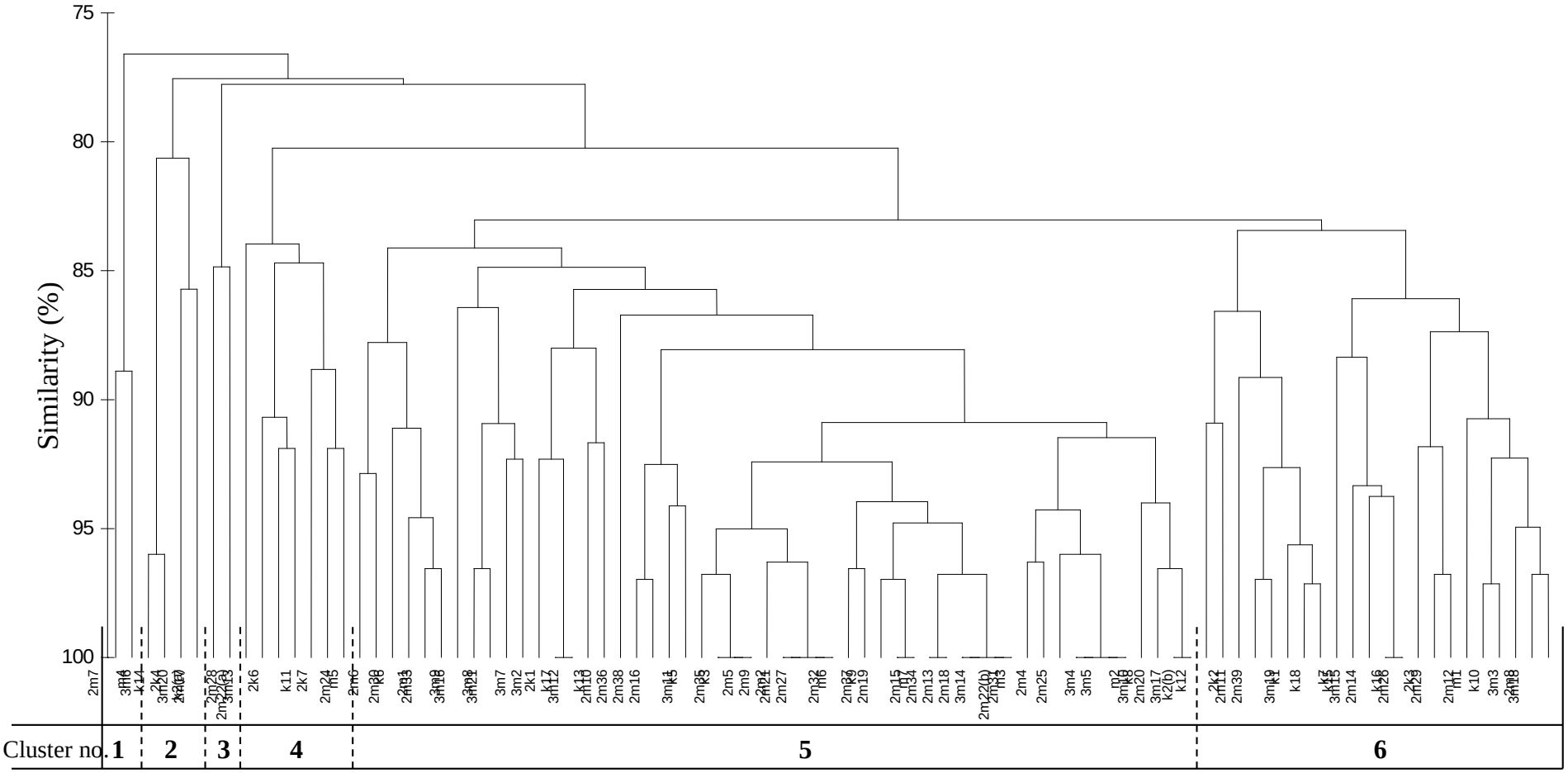

Figure 6c Cluster Analysis (CA) dendrogram showing a similarity of amplified PCR bands based on the absences/ presence of bands CGA. The letter in the labels refers to the region (e. g. M6, M= Caprivi region, 6= sample six, K= Kavango region)

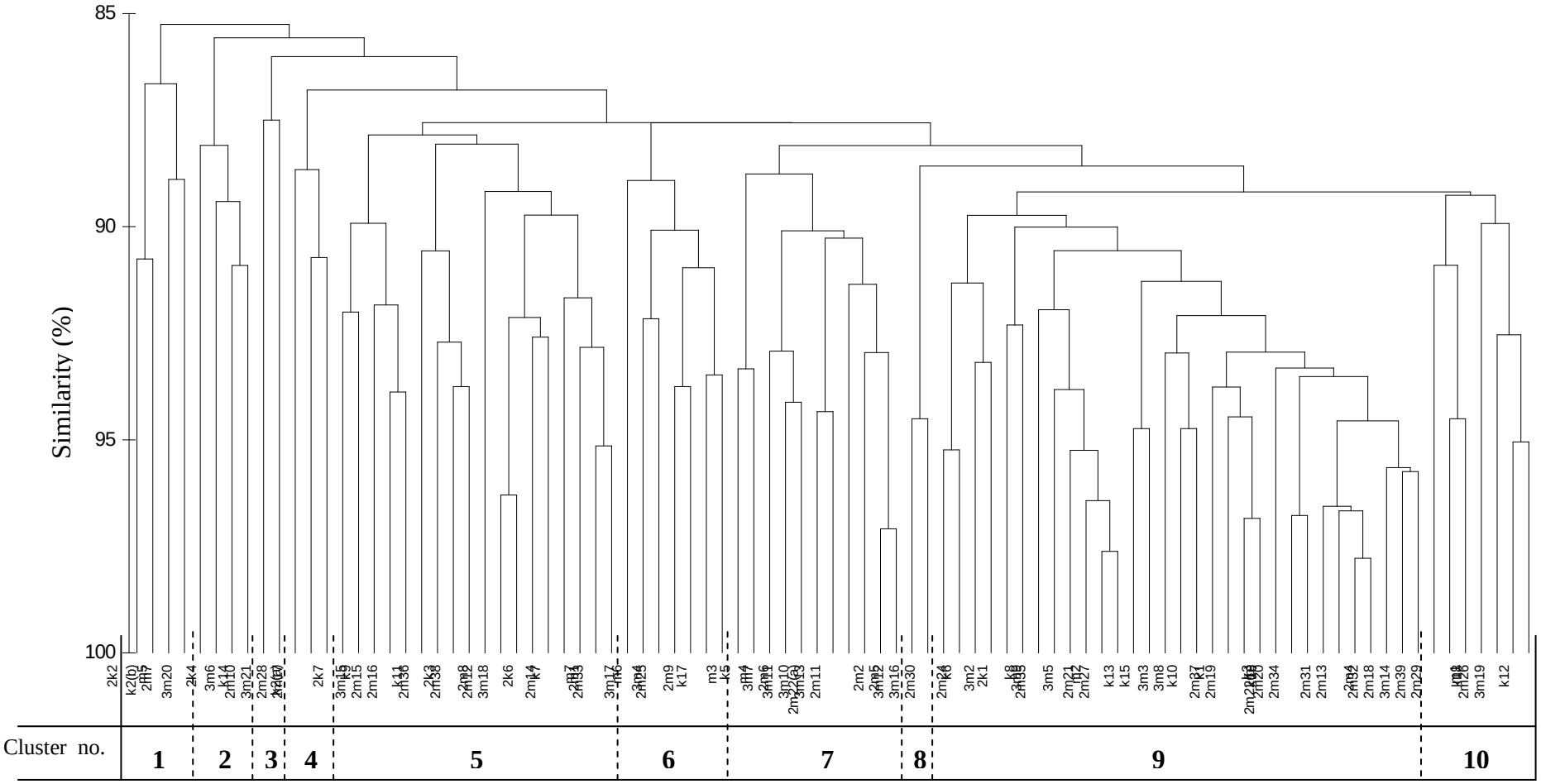

Figure 7. Cluster Analysis (CA) dendrogram of amplified PCR bands based on the absences / presence of bands all the three Primers combined. The letter in the labels refers to the region (e. g. M6, M= Caprivi region, 6= sample six, K=Kavango region)

The average within population genetic diversity of the two *Ganoderma* populations per primer ranged from 1.95 to 2.48. Primer ACA had the lowest average within population genetic diversity (1.95) while primer CGA had the highest average within population genetic diversity (2.48) see table 4 below. The average genetic diversity across three different primers was the same (2.16) for both Caprivi and Kavango region. Thus the average within population genetic diversity of *Ganoderma* in the north-eastern part of Namibia was found to be 2.16.

Table 4. Average Genetic diversity of *Ganoderma* species in the north-eastern parts of Namibia

	Primers			**Average genetic diversity**
Population	**ACA**	**CCA**	**CGA**	
Caprivi region	1.9689	2.11603	2.39995	2.16
Kavango region	1.93718	1.97461	2.55378	2.16
Average population Diversity	**1.95**	**2.05**	**2.48**	

Figure 8 revealed variation accounted for the first most two important dimensions: Principal coordinate 1 (PC1) and Pricipal Coordinate 2 (PC2). *Ganoderma* isolates were clearly separated by the first two principal Coordinates (1 and 2) with Eigen values of 63.42 and 46.45 which accounted for a total variation of 12.20 % and 8.93 % for Principal Cordinate 1 and 2 respectively (See Appendix 3). On the biplot each dot represents an individual while each band or variable is a direction used to measure the location of each point in relation to the two dimensions separately.

Thus, clumped dots indicate high genetic similarity between individuals. In other words a set of genetically similar individuals correspond to an aggregation of points on the biplot. Most *Ganoderma* isolates appeared scattered on the outer part of the quadratic area which indeed exhibit the presence of genetic variation with few clumped isolates in some quadrats that exhibit narrow or little genetic variation.

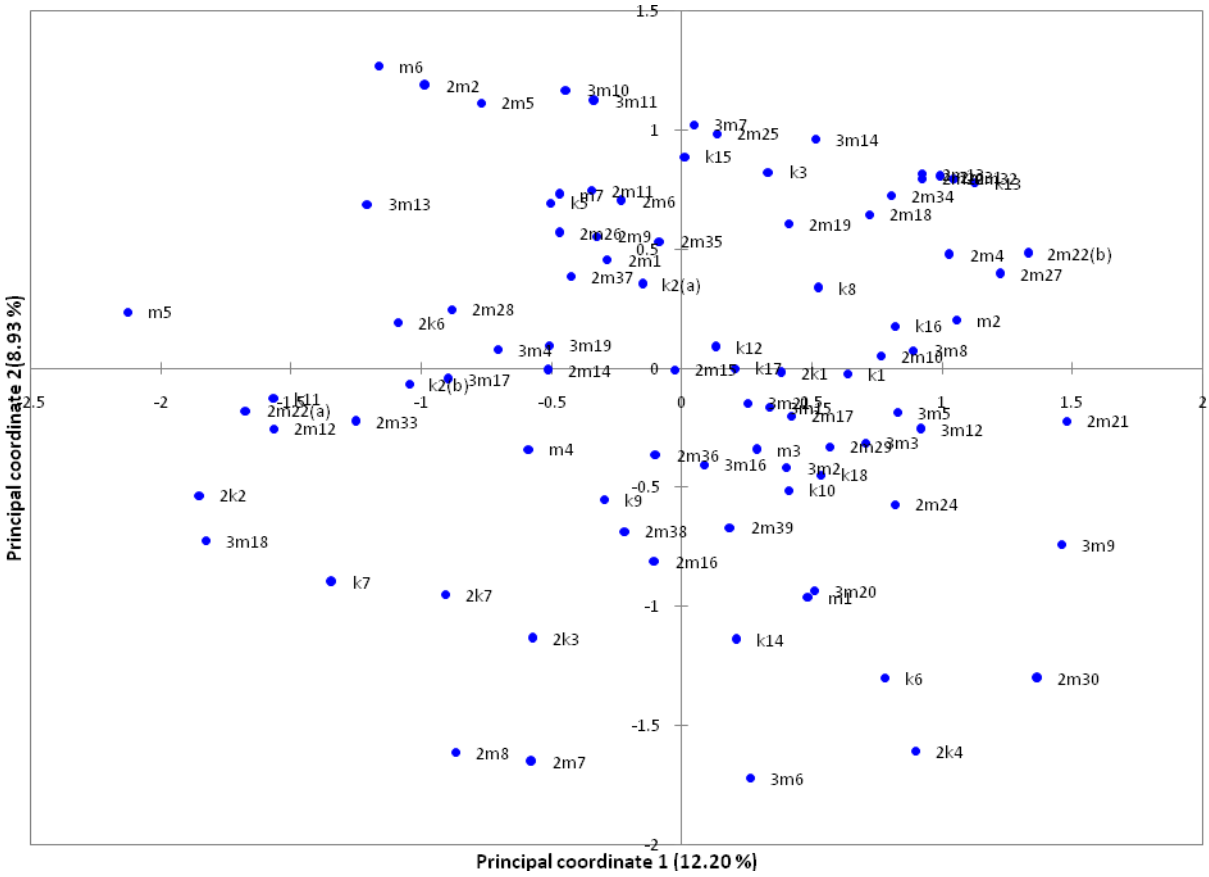

Figure 8. The first two principal coodinates of 89 *Ganoderma* isolates based on the presence and absence of RAMS data obtained in this study.

CHAPTER 5

5. Discussion

5.1 *Ganoderma* uses

5.1.1 *Ganoderma* medicinal Uses

About 90% of all the people who filled the questionnaires indicated that only *Ganoderma* caps collected from the base of stumps or live *Acacia erioloba* and *Combretum imberbe* are used medicinally. *Ganoderma* collected from *A. erioloba* and *C. imberbe* are used to halt nose bleeding, boost mother and fetus immunity during pregnancy and lastly to strengthen infant head bones. The study carried out by McMeekin (2004) found out that the medicinally important *G.lucidum* is usually found at the base of the stumps or on the roots of a living hardwood. This is very similar to the above mentioned findings of this research.

Beside the indigenous medicinal use of *Ganoderma* by local people in the north-eastern part of Namibia, fungi of the same genus have also been used in folk medicine for health promotion in Asia (Wesser, 2005; Habijanic and Berovic, 2000). Liu (1993) cited in McMeekin (2004) noted that *G.lucidum* medicinal properties have been tested on both mice and several human diseases and have indeed demonstrated medicinal properties. McMeekin (2004) stated that eastern scientists had reported that *Ganoderma* extract had in fact prolonged life spans, stimulated and regulated immune systems as well as enhanced the endocrine system activity in mice. In fact this further proved the presence of anti aging agents in *Ganoderma* extract

(Xiao et al., 1993 cited in McMeekin, 2004). Consequently this highlighted the need for further detailed study to research the nature as well as the mode of action of *Ganoderma* extract in the human body.

5.1.2 Preparation of *Ganoderma* extract

Different villagers indicated that *Ganoderma* can be prepared in different ways before use as medicine. *Ganoderma* caps can be ground to powder and then that powder is rubbed on infant's forehead in order to strengthen the skull bone. *Ganoderma* caps can also be soaked in boiled water for few minutes until the water has turned reddish. This so called *Ganoderma* tea is then taken by pregnant women as an immune booster for both mother and the fetus. This is supported by Arora (1986) cited in McMeekin (2004) who reported that since the mushroom is too tough to be edible, in Thailand *G. lucidum* is soaked in water for several months and then the extracted remedy or wine is drunk or put in a candy. Apart from the traditional ways, nowadays modern *G. lucidum* tea is sold in the markets in Thailand for medicinal purposes. *Ganoderma* can also be prepared by burning a *Ganoderma* fruiting body on a clean surface and the generated smoke can be inhaled by a nose bleeding individual to stop the nose bleeding.

5.2 *Ganoderma* hosts

Apart from unidentified hosts, trees from which most of the samples were collected where *Colophospermum mopane* (18%), *Terminalia serecia* (13%) *and Combretum collinum* (7%). *Colophospermum mopane* is one of the dominant woody trees of the woodland biome of Namibia of which the Kavango and Caprivi region are part of

(Mfune, 2005). According to Erkel (2009) *Ganoderma* species favours hosts with high carbohydrate contents for energy source as well as adequate protein source for formation of fruiting bodies. Erkel (2009) further elaborated that, they do not favour situation where there is a lack of protein, hence there is a need to convert carbon to protein. Carbohydrate is indicated in Erkel (2009) to have a major role in *Ganoderma* species as it is the main source of energy and carbon during the growth.

Ganoderma species are generally associated with solid hosts that have high moisture contents, therefore normally abundant in very humid places (Habijanic and Berovic, 2000). Lack of adequate moisture is associated with reduction of polysaccharides production and mycelia growth (Habijanic and Berovic, 2000). Beside humid environmental conditions, the moisture holding capacity of a particular woody material also tends to play a major role in determining the presence or absence of *Ganoderma* on a particular woody host. Polysacharides seem to have two main roles namely: to fasten the hyphae to the surface of the host as well as protecting the hyphae against evaporation and mechanical disturbance (Habijanic and Berovic, 2000). For this reason the availability of few number of *Ganoderma* on some woody trees can be perhaps partially explained by the presence of inadequate amount of polysaccharides in the host.

Apart from nutrient composition of the hosts, other factors such as sampling techniques, age of the hosts and variation in microhabitats also play a part in the presence or absence of *Ganoderma* on various hosts. The fungi disperse their spores by wind (Elliot and Paschot, 2000) which presents equivalent chances of host

colonization. Variations in microhabitats temperature, humidity of different habitats, host specificity of different *Ganoderma* species and geographical location of different trees tends to play a major role in the determination of the spores germination. Although this study only focused on the appearance of fruiting bodies as the presence of *Ganoderma* on hosts, Elliot and Paschot (2000) reported that the fungi may still be present inside the host as hyphae.

In addition, higher number of *Ganoderma* collected from *Colophospermum mopane* can also be explained by the fact that most of these samples were collected from homesteads especially in the Caprivi region in which *Colophospermum mopane* posts are commonly used as building Materials. Accessibility of different woodlands affects the hosts as some trees are found in fenced areas and not found in homesteads. This might have affected the frequency of some hosts in this research.

It can clearly be seen in Table 3 that 4% of the samples collected in this research were found on soil hosts (Appendix 1a). Even though analysis of soil samples from which the samples were not conducted in this study, Wasser (2005) had reported the same findings. Wasser (2005) further reported that *Ganoderma* fungi can be occasionally found on soils either arising from buried roots or stumps and also on soils that have high concentration of decomposing wood debris or wood chips.

Table 3 also exhibit the state of the tree host on which *Ganoderma* samples were collected; which were grouped as either dead stump, dead burned stumps (Appendix 1b) including building posts (Appendix 1d) or dead unburned stumps (Appendix 1c)

or live trees. Even though *Ganoderma* is commonly found on dead trees or sprouting stumps, Elliot and Broschat (2000) confirmed that indeed some species of *Ganoderma* can be found on live trees, the same results were obtained in this study. Elliot and Broschat (2000) supported this idea by giving evidence of *G. lucidum* as the common cause of white rot disease in live oak trees in Florida. However this can not be used as sole identification tools for *G. lucidum* since other *Ganoderma* species might also colonize live trees of different species in different areas. For all tree host recorded in this study *Ganoderma* isolates were only found either on the root or within 122cm to 152cm on the tree trunk from the soil surface but not found on branches higher up on the trees.

5.3 Genetic diversity and PCR amplification

A high within population genetic variation of an average was found for both populations from Kavango and Caprivi region using Shannon Wiener Index resulting in an average of 2.16 for genetic diversity of *Ganoderma* species in the north-eastern parts of Namibia. Genetic heterogeneity could also be seen clearly through the presence of various band sizes in all the gels pictures (Figure 5a, 5b, 5c), in dendrograms, most pronounced in the dendrogram of CGA (Figure 6c) and also in combined primers dendrogram (Figure 7) analysis which resulted in a highly branched dendrograms.

The formation of multiple branches or stems at different significance level in dendrograms indeed indicated the presence of variation in sequence of interest and

number of repeats of used primers in different *Ganoderma* isolates. Beside these, another form of genetic diversity was also exhibited by the absence of amplified bands in some individuals which may indicate the absence of the complementary sequences of the primer used (Figure 5a; well 4, 6, and Figure 5b; well 7, 18, 19). Alternatively, diversity is not the sole explanation for absence of bands in samples as it can also be accounted for by the size and reproducibility of the resultant PCR product.

According to Mohammadi and Prasanna (2003) rareness of aggregation of individuals as the results in Figure 8 in fact revealed high genetic variation between *Ganoderma* isolates in the north-eastern parts of Namibia, therefore confirmed the results of the highly branched HCA cluster in Figure 8 as well as conclusions made by Shannon-Wiener index of diversity. In relation to the first two dimensions most *Ganoderma* isolates were scattered from each other with total variation of 12.20 % and 8.93 % respectively. This graph mainly indicated the degree of similarity which can be seen by the proximity of individuals on the PCoA biplot. Hence the proximity of individuals in fact confirmed genotype variation among *Ganoderma* isolates (Demey et al., 2008) (Figure 8).

It should be noted however, that specific alleles that accounted for high total variation revealed in the consequential genotype ordination of *Ganoderma* isolates can not be identified. This is mainly because none of the tests used in this study could accomplish that. Demey et al. (2008) clearly indicated that PCoA results can not be interpreted in relation to the original variables (bands) mainly because PCoA

axis has no direct meaning. Demey et al. (2008) further explained that PCoA only creates biplots on which geometrical distance between points on the plot reflect genetic distances between individuals with reduced distortion. In fact PCoA visualize the genotype differences among individuals and form possible groups or aggregates (Mohammadi et al., 2003)

Although high overall genetic diversity was observed, there were also some *Ganoderma* isolates which indicated 100% similarity commonly in Primer CCA (Figure 6b) and Primer ACA (Figure 6a) respectively. This can be explained by the fact beside sexual reproduction, basidiomycetes also reproduce asexual even though less common than sexual reproduction. Basidiomycetes reproduce both asexually and sexually (Campbell and Reece, 2005). Furthermore, asexually reproduction employs mitosis cell division resulting into genetically identical offspring, 100% genetic similarity.

The banding pattern produced by each of the three primers was highly variable (Figure 6a; 6b; 6c). In addition most amplified bands produced by these primers were polymorphic. Polymorphic results obtained in this study in fact, show the usefulness and the sensitivity of these random amplified microsatellites in genetic studies of fungi. The same findings were reported by Hantula et al. (1996). Hantula et al. (1996) added that RAMS are very reproducible and tend to be the best methods in detection of polymorphism when compared to other methods. The presence of numerous polymorphic bands in PCR results of all the three primers used in this

study indicate the existence of these microsatellites in large quantities in *Ganoderma* species (Zakariah, et al., 2005) in the north-eastern parts of Namibia.

According to Hantula et al. (1996) there is no certainty about the source of variation in RAMS, however it is usually associated with three different hypothesis. The first one is that mutation at priming sites could prevent fragment amplification as in RAPDs, although the latter is less likely in microsatellites as the variability is mostly caused by differences in number of repeats (Charlesworth et al., 1994 cited in Hantula et al., 1996). Secondly, the outcome of insertion or deletion events may either result in a length polymorphism or absence of a PCR product depending on the reproducibility of the resultant fragment size (Hantula et al., 1996). Hantula et al. (1996) further outlined that the third assumption is that variation in the number of microsatellites repeats may result in size polymorphism.

Hantula et al. (1996) clearly pointed out that when the same RAMS primers were used on *Ganoderma* in America and Europe, the sequence result of some primers contained short segments of other primers within them. Certainly this indicates that amplified regions may be rich in more microsatellites beside the one of the specific primer used. However, this could not be tested in this study as none of the PCR results were sequenced.

Even though the major source of genetic variation in this population is uncertain, usually high levels of genetic variation are expected in sexually reproducing organism as a result of crossing over of genetic materials in meiosis prior to the

formation of gametes (Campbell and Reece, 2005; Mader, 2004, Zakariah et al., 2005). Additionally, Zakariah (2005) further stated that genetic diversity within *Ganoderma* species can be a result of adaptation to wide various geographical regions, adaptation to exploit different hosts or maybe the genus had evolved from an ancestor with a wide genetic base. Lastly, high level of genetic diversity in *Ganoderma* species perhaps maybe a result of horizontal gene transfer which usually happens between pathogens and their host (Lawrence, 2005). Through natural selection pathogens tend to evolve relative ways of gene acquisition from their hosts for better adaptation to their hosts (Lawrence, 2005) and as mentioned earlier some *Ganoderma* species can be pathogens at some stages of their life cycle.

CHAPTER 6

6. Conclusion and Recommendation

6.1 Conclusion

To conclude, the study only focused on the presence of *Ganoderma* fruiting bodies as the presence of *Ganoderma* species on hosts. All three primers (ACA, CCA and CGA) used in this studies as well as dendrograms constructed by Primer 5 exhibit the existence of a high degree of genetic diversity within genus *Ganoderma* in the two regions of the north-eastern parts of Namibia. This study suggests that the average genetic diversity between *Ganoderma* isolates in the north-eastern parts of Namibia is 2.16. First two dimension of PCoA accounted for 12.20% and 8.93% total variation respectively, with Eigen value of 63.42 and 46.45 respectively. The study also supports the usefulness of RAMS in detecting polymorphism and the role it plays in fungi genetic diversity studies. Conclusions could be made that RAMS are highly informative in genetic diversity studies of genus *Ganoderma*. The use of both Cluster Analysis and PCoA presented improved genotype ordination results, since they produce different clusters in relation to the two most important coordinates and also measure the quality of the resultant ordination. Further conclusions could be made that *Ganoderma* is only used for three medicinal purposes by indigenous people of the north-eastern parts of Namibia. These are to halt nose bleeding, strengthening of infant head bones and immune boosting. Thirteen natural hosts of *Ganoderma* species were identified consisting of 12 tree species and soil in the north-eastern part of Namibia.

6.2 Recommendations

6.2.1 Prior assessment of the area before actual sampling

Prior assessment is very crucial in marking the various numerous specific locations where isolates are found, so that during sampling time collectors will go straight to the identified locations. This will ease the entire sampling process in a way that all the time allocated for sampling will be spent on actual sampling other than most of the sampling time spend on searching for *Ganoderma* population in the forest and gathering knowledge on *Ganoderma* distribution which would result alteration of sampling methods. Beside this it is also necessary to assess which areas are accessible for sampling. This is because some areas are protected by traditional doctors and some have dense populations of Venomous snakes. All these limited the number of samples that were collected and also excluded certain areas from sampling.

Pre assessment of the area would also help in isolating different population that can be used in the study so that in the future genetic diversity should be compared between different distinct populations of *Ganoderma* in a particular region. In this study it was hard to spot *Ganoderma* population in the forest. It is hereby recommended that prior assessment of the sampling area should be done in order to improve the sampling process and also to ease the formulation of the appropriate sampling method.

6.2.2 The use of specific Primers

Primers that were used in this study were generated for broad use in three different families: Ascomycetes, Phycomycetes and Basidiomycetes and not specifically for *Ganoderma* species. In addition to this the primers were designed specifically for fungi in America and Europe. To improve the findings of the study it is recommended that specific primers for the Namibian *Ganoderma* population should be used in any molecular study targeting the Namibia *Ganoderma* as the focus of the study. These primers should be developed by screening the Namibian *Ganoderma* germplasm.

6.2.3 The use of laboratory tissue lysers for sample grinding

Ganoderma has a very tough texture, making it very difficulty to grind with a mortar and pestle. It is advisable to use a tissue lyser to grind *Ganoderma* samples prior to DNA extraction. This will reduce the chances of cross contamination between samples and enhance the quality of extracted DNA as the machine grind finer, thus create a larger surface area.

6.2.4 Further examination of PCoA figures

In the future it is highly recommended that binary matrices for genotype data which are normally obtained by employing molecular markers should be analysed by using a combination of PCoA, CA and External Logistic Biplots (EBL). This will ensure determination of the allele that is represented in the principal coordinate axis as most contributing variation factor and thus enabling allele to allele comparison the latter

individual to individual comparison. Further breakdown of PCoA figures will also ease the interpretation of genotype classification results as specific bands responsible for genetic classification can be identified.

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Appendices

Appendix 1a. *Ganoderma* on soil hosts

Appendix 1b. *Ganoderma* isolate growing on a dead burned woody stump

Appendix 1c. *Ganoderma* isolates growing on a dead unburned woody stump**s**

Appendix 1d. *Ganoderma* isolates on building posts

	1																	19										30											43
2k1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0
2k2	0	1	0	0	1	1	1	0	1	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	1	1	1	0	0	0	1	1	0	1	1	1	0	0	1	0	0	0
2k3	0	1	0	1	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2k4	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2k6	0	1	1	1	1	1	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0
2k7	0	0	1	1	1	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
2m1	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
2m10	0	1	1	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0
2m11	0	1	1	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0
2m12	0	1	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
2m13	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
2m14	0	1	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0
2m15	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
2m16	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
2m17	0	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
2m18	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
2m19	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
2m2	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0
2m20	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0
2m21	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2m22(a)	0	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0
2m22(b)	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
2m24	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
2m25	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
2m26	0	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
2m27	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
2m28	0	0	1	1	0	0	1	1	1	0	1	1	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0
2m29	0	1	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0
2m30	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2m31	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
2m32	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0

Appendix 2. Presence (1) and absence (0) matrix of PCR bands developed by primer CGA (lanes 1-19), primer CCA (lanes 20-30)and primer ACA (lanes 31-43)

	1																		19										30										43
2m33	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0
2m34	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0
2m35	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
2m36	0	1	1	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
2m37	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0
2m38	0	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2m39	0	1	1	0	1	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0
2m4	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
2m5	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
2m6	0	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0
2m7	0	1	0	0	1	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2m8	0	1	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0
2m9	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0
3m10	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0
3m11	0	1	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	0	1	1	0	1	0	0	1	0	0	0
3m12	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
3m13	0	1	1	1	1	0	0	1	1	0	1	1	0	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0
3m14	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
3m15	0	1	0	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0
3m16	0	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0
3m17	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0
3m18	0	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0
3m19	0	1	1	0	1	1	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0
3m2	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
3m20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3m21	0	0	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0
3m3	0	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
3m4	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
3m5	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3m6	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
3m7	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	0	0	0	0

	1																		1 9											3 0									4 3
3m8	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0
3m9	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k1	0	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
k10	0	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
k11	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	1	1	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0
k12	0	1	1	1	1	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0
k13	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0
k14	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
k15	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0
k16	0	1	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0
k17	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
k18	0	1	1	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0
k2(a)	0	0	1	1	0	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
k2(b)	0	1	0	0	1	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0
k3	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0
k5	0	1	1	1	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0
k6	0	0	1	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k7	0	1	0	1	1	1	1	1	1	1	0	1	0	0	0	0	1	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
k8	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0
k9	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0
m1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
m2	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
m3	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
m4	0	1	1	0	1	1	0	1	1	1	1	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0
m5	0	1	1	1	1	0	1	1	1	1	1	0	0	1	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	1	0	1	1	1	1	1	1	0	0	1	0	0	0	0
m6	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	1	0	1	1	1	1	1	1	0	1	1	0	0	1	0
m7	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0

Appendix 3. Calculated Eigen values for the first six PCoA axes

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	63.423	46.454	40.410	30.815	26.928	26.168
Variability (%)	12.197	8.934	7.771	5.926	5.179	5.032
Cumulative %	12.197	21.131	28.903	34.829	40.008	45.040

Appendix 4. The questionnaire used in this study

Questionnaire

Region_____

Name of the village_____

Sex of the respondent Male ☐ Female ☐

1. Do you know mushrooms? Yes ☐ No ☐

2. If yes what types of mushrooms?

_____

_____

_____

_____

_____

3. Do you eat mushrooms? Yes ☐ No ☐

4. If yes what types of mushrooms do you eat?

_____

_____

_____

_____

5. How often do you collect mushrooms and who collect them?

_____

_____

6. Do you use *Ganoderma* extract for medicinal purposes? YES NO

7. Specifically state the illnesses that you treat with *Ganoderma.*

8. How do you harvest *Ganoderma* mushrooms?

9. How do you store and prepare *Ganoderma* extract before use?

Appendix 5. Date, latitudes and longitudes where and when the samples were collected for this study

Date	Latitudes	Longitudes	Feet
3/3/2010 10:41	S18 18.890	E19 22.504	3822 ft
3/3/2010 11:28	S18 14.533	E19 26.391	3828 ft
3/3/2010 12:18	S18 07.823	E19 32.492	3790 ft
3/3/2010 12:54	S18 03.212	E19 37.966	3797 ft
3/4/2010 9:30	S17 52.986	E19 35.416	3549 ft
3/4/2010 9:44	S17 53.011	E19 35.371	3516 ft
3/4/2010 13:59	S17 56.213	E19 56.154	3524 ft
3/4/2010 14:03	S17 56.216	E19 56.155	3507 ft
3/4/2010 14:37	S17 56.280	E19 56.409	3606 ft
3/4/2010 16:04	S17 54.778	E20 06.826	3582 ft
3/5/2010 8:37	S18 03.512	E19 37.576	3572 ft
3/5/2010 8:45	S18 03.492	E19 37.626	3684 ft
3/5/2010 8:52	S18 03.565	E19 37.615	3686 ft
3/5/2010 9:01	S18 03.574	E19 37.606	3732 ft
3/5/2010 9:10	S18 03.657	E19 37.572	3735 ft
3/5/2010 10:58	S18 09.509	E19 31.008	3720 ft
3/5/2010 11:13	S18 10.505	E19 30.159	3742 ft
3/5/2010 12:44	S18 13.584	E19 26.976	3743 ft
3/5/2010 12:50	S18 13.604	E19 26.966	3828 ft

3/5/2010 13:00	S18 13.643	E19 26.950	3840 ft
3/6/2010 15:47	S17 42.290	E24 00.107	3534 ft
3/6/2010 16:03	S17 41.726	E24 01.926	3187 ft
3/6/2010 17:17	S17 31.774	E24 16.125	3118 ft
3/6/2010 17:22	S17 31.774	E24 16.125	3117 ft
3/6/2010 18:13	S17 30.076	E24 16.321	3113 ft
3/7/2010 11:08	S17 33.422	E24 21.992	3110 ft
3/7/2010 11:11	S17 33.431	E24 21.993	3112 ft
3/7/2010 11:16	S17 33.452	E24 21.980	3089 ft
3/7/2010 11:17	S17 33.423	E24 21.976	3076 ft
3/7/2010 11:52	S17 33.104	E24 22.877	3079 ft
3/7/2010 12:34	S17 35.036	E24 18.706	3077 ft
3/7/2010 12:56	S17 32.982	E24 17.350	3080 ft
4/6/2010 12:52	S17 54.834	E20 25.729	5549 ft
4/6/2010 12:59	S17 54.805	E20 25.727	5547 ft
4/6/2010 13:05	S17 54.805	E20 25.728	5549 ft
4/6/2010 13:11	S17 54.803	E20 25.723	5549 ft
4/6/2010 13:22	S17 54.755	E20 25.737	5545 ft
4/6/2010 13:52	S17 54.577	E20 25.855	5524 ft
4/8/2010 8:52	S17 30.715	E24 16.081	5546 ft
4/8/2010 8:52	S17 30.713	E24 16.081	5547 ft
4/8/2010 8:52	S17 30.716	E24 16.085	5547 ft
4/8/2010 9:02	S17 30.684	E24 16.087	5548 ft

4/8/2010 9:21	S17 31.784	E24 16.454	5547 ft
4/8/2010 9:27	S17 31.795	E24 16.448	5535 ft
4/8/2010 9:56	S17 32.496	E24 17.223	5548 ft
4/8/2010 9:58	S17 32.500	E24 17.253	5551 ft
4/8/2010 10:19	S17 32.496	E24 17.191	5549 ft
4/8/2010 10:24	S17 32.495	E24 17.185	5550 ft
4/8/2010 10:27	S17 32.499	E24 17.215	5533 ft
4/8/2010 10:43	S17 32.786	E24 17.383	5547 ft
4/8/2010 10:57	S17 32.787	E24 17.505	5547 ft
4/8/2010 11:07	S17 32.738	E24 17.541	5548 ft
4/8/2010 11:23	S17 32.804	E24 17.568	5550 ft
4/8/2010 11:29	S17 32.804	E24 17.555	5550 ft
4/8/2010 11:29	S17 32.804	E24 17.545	5550 ft
4/8/2010 11:33	S17 32.832	E24 17.558	5548 ft
4/8/2010 11:41	S17 32.828	E24 17.575	5551 ft
4/8/2010 11:59	S17 35.139	E24 18.535	5551 ft
4/8/2010 12:04	S17 35.136	E24 18.539	5543 ft
4/8/2010 12:16	S17 35.168	E24 18.502	5548 ft
4/8/2010 12:16	S17 35.164	E24 18.500	5549 ft
4/8/2010 12:16	S17 35.167	E24 18.497	5551 ft
4/8/2010 14:09	S17 28.768	E24 14.874	5550 ft
4/8/2010 14:48	S17 34.945	E24 22.239	5549 ft
4/8/2010 15:05	S17 35.585	E24 22.776	5546 ft

4/8/2010 15:08	S17 35.582	E24 22.778	5551 ft
4/8/2010 15:09	S17 35.578	E24 22.777	5550 ft
4/8/2010 15:30	S17 36.331	E24 23.237	5551 ft
4/8/2010 15:31	S17 36.329	E24 23.234	5548 ft
4/8/2010 15:34	S17 36.373	E24 23.254	5544 ft
4/8/2010 15:35	S17 36.367	E24 23.260	5547 ft
4/8/2010 15:35	S17 36.367	E24 23.260	5549 ft
4/8/2010 15:39	S17 36.353	E24 23.258	5542 ft
4/8/2010 15:39	S17 36.350	E24 23.260	5547 ft
4/8/2010 16:04	S17 37.573	E24 24.331	5495 ft
4/8/2010 16:06	S17 37.571	E24 24.332	5549 ft
4/8/2010 16:06	S17 37.571	E24 24.330	5547 ft
4/8/2010 17:20	S17 35.099	E24 22.422	5551 ft
5/13/2010 16:43	S17 43.037	E24 32.453	5539 ft
5/13/2010 17:07	S17 43.082	E24 37.018	5549 ft
5/13/2010 17:12	S17 42.768	E24 36.913	5551 ft
5/13/2010 17:12	S17 42.768	E24 36.922	5550 ft
4/15/2010 16:48	S17 42.759	E24 36.952	5547 ft
5/13/2010 17:51	S17 45.017	E24 40.983	5550 ft
4/15/2010 16:53	S17 45.018	E24 40.983	5550 ft
5/13/2010 17:55	S17 45.435	E24 40.845	5551 ft
4/16/2010 9:53	S17 45.435	E24 40.846	5550 ft
4/16/2010 10:02	S17 45.435	E24 40.846	5550 ft

4/16/2010 10:12	S17 45.435	E24 40.846	5551 ft
5/14/2010 12:09	S17 46.298	E24 35.265	5548 ft
4/16/2010 11:58	S17 46.296	E24 35.262	5548 ft
4/16/2010 12:50	S17 46.296	E24 35.262	5550 ft
5/14/2010 13:56	S17 52.977	E24 40.514	5548 ft
5/14/2010 13:56	S17 52.978	E24 40.512	5548 ft
5/14/2010 13:56	S17 52.981	E24 40.508	5547 ft
5/14/2010 14:34	S17 45.672	E24 30.496	5551 ft
5/14/2010 14:34	S17 45.674	E24 30.499	5550 ft
5/14/2010 15:35	S17 52.992	E24 23.797	5549 ft
5/14/2010 16:08	S17 52.988	E24 23.791	5551 ft