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Genetic Diversity of *Ganoderma* Species in the North Eastern Parts of Namibia using Random Amplified Microsatellites (RAMS)

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Polypore fungi are characterized by the presence of pores instead of the gills on the lower side of the fruiting body. Genus *Ganoderma* is one of the polypore fungi. Species within this genus possess high resemblance making, thus species level identification is very challenging. The objective of this study was to determine the genetic diversity among *Ganoderma* species in the north-eastern part of Namibia. The study was carried out in two regions; Kavango and Caprivi. A total of 89*Ganoderma* samples were used for PCR amplification. DNA extraction was done using Zymo Research (ZR) Bacterial/ Fungal DNA kit. The genetic diversity was determined by using three different random amplified microsatellites primers. Shannon-Weiner Index of diversity, Cluster Analysis (CA) and Principal Coordinate of Analysis (PCoA) were the three numerical classification methods used to analyze RAMS data. 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. Lastly high genetic diversity of *Ganoderma* can be explained by various factors including sexual reproduction host specificity, adaptation to different environmental conditions and gene transfer between pathogens and hosts.

Key words: *Ganoderma*, genetic diversity, Namibia, RAMS.

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.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.,*

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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 or extraction.

Furthermore, 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. Not only that, but also the capability to be able to identify a certain fungi by using DNA sequences only 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 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).Beside tremendous effort by various researchers to identify *Ganoderma* species using isoenzymatic studies, molecular data as well as morphological data,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*.

This study focused on genetic diversity as a measure of biodiversity in the genus *Ganoderma*. In most cases due to the complexity of the genus like *Ganoderma*, genetic diversity analyses are usually 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). Analysis of genetic diversity is very crucial for the exploration of the medicinal value of *Ganoderma* species.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).

MATERIALS AND METHODS

The samples were collected from north-eastern part of Namibia which was divided into two regions: Kavango and Caprivi. 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 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. Extraction was done following the ZR instruction manual.

PCR amplification

Polymerase Chain Reaction (PCR) was used for DNA amplification using threedifferent 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*™ 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*™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). 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*™ 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°C, CGA was 61°C and for ACA was 49°C. The amplification processes was finished with a single final extension at 72°C for 7 minutes. All PCR amplifications were done using a Bio-Rad Thermal Cycler.

Gel electrophoresis

Fermentas Dream *Taq*™ 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 then stained with 1 µl per 100 ml of Ethidium bromide and it was viewed under UV fluorescent light andthen 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.

Data analysis

The PCR products were scored absence (0) and presence (1) for non-amplification and amplification fragments and recorded in a binary matrix.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) adapted from Smith,(1990); Monaghan and Halloran, (1996) 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).All PCoA analyses were also calculated using XLSTAT 2011 software (Lücking *et al.,* 2011).

RESULTS

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. Fig. 1 below shows the amplified bands of different *Ganoderma* samples (well 2 to well 19) using primer ACA, in comparison to the 100 bp DNA ladder in the first well (1).

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. The average similarity values of these RAMS widely range from 85.5% to 98.2%.

Table 1. Average genetic diversity of *Ganoderma* species in the north-eastern parts of Namibia

Population	Primers			Average
	ACA	CCA	CGA	genetic diversity
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	

The combined primers' dendrogram was divided into 10 clusters as indicated in Fig. 2. Even though numerous clusters were formed by each primer during the Cluster Analysis (CA), none of the analysis (Fig. 2) exhibit *Ganoderma* isolates from Kavango region clustering separately from *Ganoderma* isolates from Caprivi 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 1 below. The average genetic diversity across three different primers was the same (2.16) for both Caprivi and Kavango regions. Thus the average within population genetic diversity of *Ganoderma* in the north-eastern part of Namibia was found to be 2.16.

Fig. 3 below 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. On the biplot each dot represents a single *Ganoderma* isolate 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

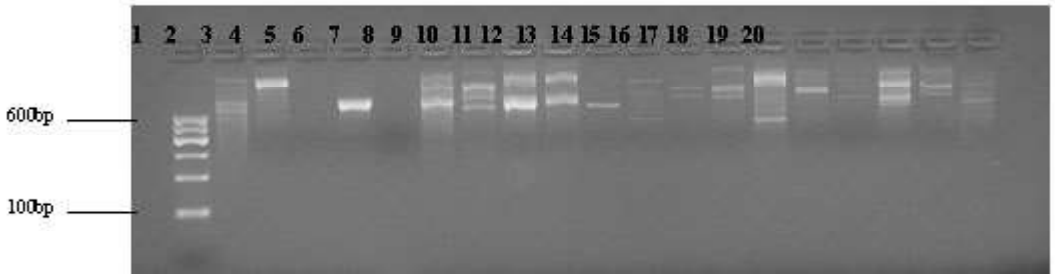

Fig. 1. PCR amplification bands of ACA primer in a 2.5 % agarose gel

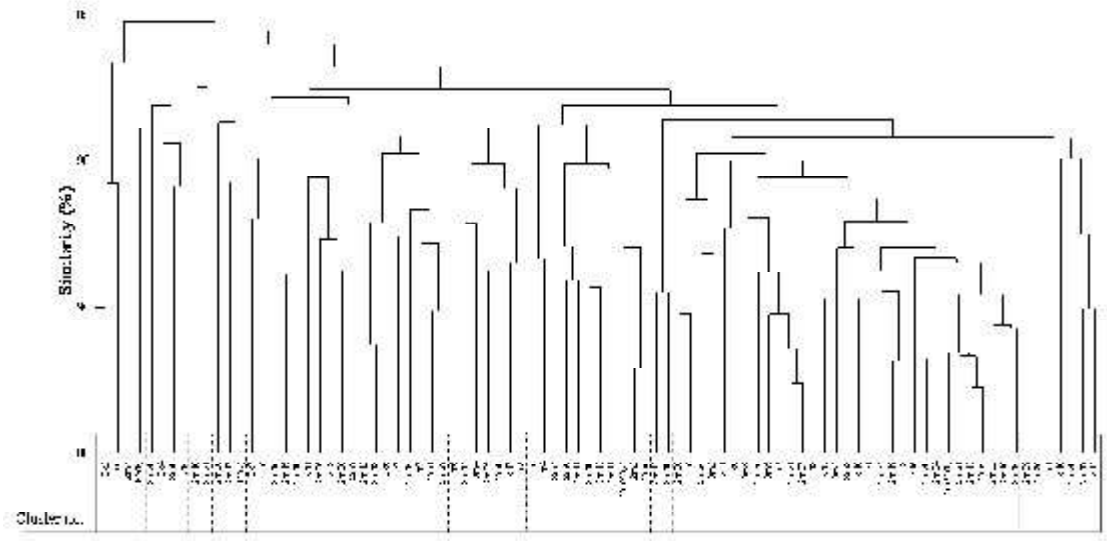

Fig. 2. Cluster Analysis (CA) dendrogram of amplified PCR bands based on the absences / presence of bands all the three Primers combined. The letter in the labels refers to the region (e. g. M6, M= Caprivi region, 6= sample six, K=Kavango region

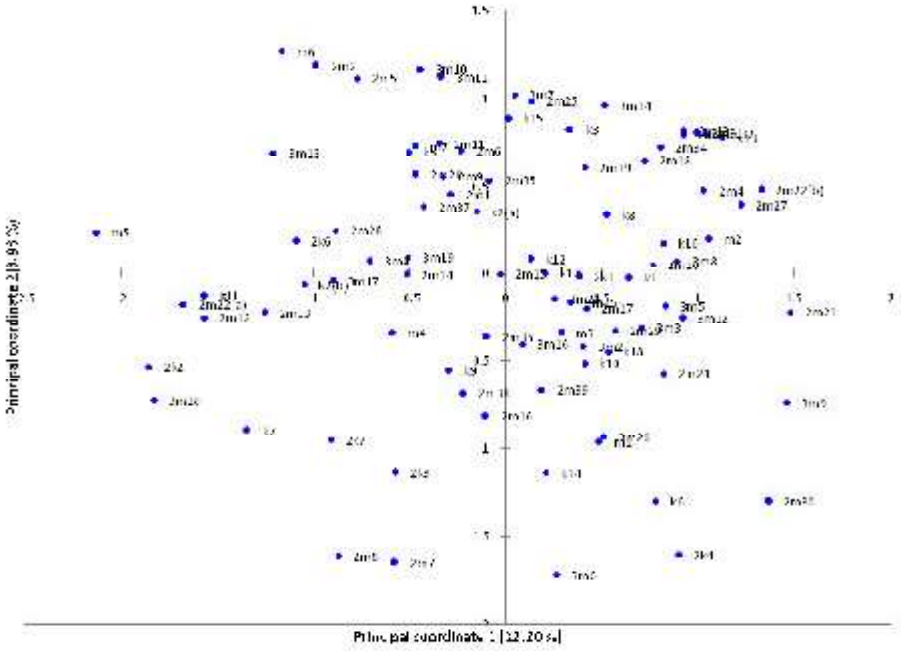

Fig. 3. The first two principal coodinates of 89 *Ganoderma* isolates based on the presence and absence of RAMS data obtained in this study

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.

DISCUSSION

A high within population genetic variation of an average was found for both populations from Kavango and Caprivi regions 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 (Fig. 1) and also in combined primers dendrogram (Fig. 2) analysis which resulted in a have numerous branches.

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 (Fig. 1; well 4, 6). 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 3 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 Fig. 3 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 individualsin fact confirmed genotype variation among *Ganoderma* isolates (Demey *et al.,* 2008) (Fig. 3).

It should be noted however, that specific alleles that accounted for high total variation revealed in the consequential genotype ordination of *Ganoderma* isolates cannot 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 cannot 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 single primers dendrograms. 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 & 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. 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 hypotheses. 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.

CONCLUSIONS

All three primers (ACA, CCA and CGA) used in this study 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.

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