CHARACTERISATION OF JATROPHA CURCAS L. GERMPLASM WITHIN BOTSWANA USING MORPHOLOGICAL AND MOLECULAR MARKERS (SSR)

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CHARACTERISATION OF *Jatropha curcas L.* GERMPLASM WITHIN BOTSWANA USING MORPHOLOGICAL AND MOLECULAR MARKERS (SSR)

A Dissertation presented to the Department of Crop Science and Production in partial fulfillment of the requirements of the Degree of Masters of Science (MSc) in Crop Science (Agronomy)

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STATEMENT OF ORIGINALITY

I, hereby declare that the work contained in this thesis was compiled by the author at Botswana University of Agriculture and Natural Resources between August 2014 and June 2017. It is original work except where due reference is made and neither has been nor will be submitted for the award of any other degree by any other University.

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DEDICATION

This work is dedicated to my wife, child and mother who have always loved me unconditionally

ABSTRACT

Jatropha curcas L. is an undomesticated plant species that has recently received great attention for its utilization in biofuel production in many countries including Botswana. The main goal of this study was to identify unique genotypes or sources of genetically diverse genotypes that will serve as parental line for *Jatropha curcas* breeding programme in Botswana.

The study was conducted at two sites at Botswana University Agriculture and Natural resources Tissue Culture Laboratory and Department of Agricultural Research Station Nursery during the period of October 2015 to May 2016. A total of *30 Jatropha curcas* accessions were used. Three trees per accessions were randomly tagged for data collection in the field. Accessions were characterized for 16 quantitative and 8 qualitative characters. The results indicated considerable variation for quantitative characters, as well as significant morphological difference in almost all the characters. The qualitative characters revealed substantial amount of variability among the accessions except in fruit shape, stem color and flower color. Multivariate cluster and principal coordination analysis based on morphological characters grouped the accessions into four clusters at dissimilarity coefficient of approximately 1.37 indicating a wider variation. Principal component analysis identified characters that accounted for the total variation. The first five principal components accumulated total variation of 71.74%. This was mostly associated with the quantitative characters. This indicates the most important characters to consider when characterization *Jatropha curcas* morphological under limited resources.

Molecular characterization involved genomic DNA isolated from young leaves of the same 30 *Jatropha curcas* accessions using CTAB DNA extraction protocol. DNA was confirmed with a Nano Drop as well 2% agarose gel with ethidium bromide staining. A total of 22 SSR primers pairs were tested with DNA sample for reproducible amplification following the touchdown 50/45 PCR program. Four markers did not amplify DNA samples for all accessions and were discarded. The scored data for 18 markers was assessed for polymorphism by calculating the polymorphic information content (PIC). PIC value for each marker was 0.00 indicating lack of informativeness. The cluster analysis revealed four distinct clusters at 0.85 genetic similarity coefficients and this indicates narrow genetic diversity. The markers that accounted for the total variation were identified through principal component analysis and were associated with four principal components which accounted for 73.91% of the total cumulative variance.

Correlation of morphological and molecular markers showed negative, positive and no correlation relationships. A total of eight morphological characters and six molecular markers correlated positively with r values ranging from 0.557 to 0.662. Whereas negative and no correlation relationship revealed by majority of the markers. This indicates that microsatellite markers indeed do not sample the same genomic regions with genes that control the expression of phenotypes.

Key words. Characterization, Genetic diversity, Morphological markers (Quantitative and Qualitative traits), Molecular markers, SSR, and *Jatropha curcas*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AFLP	Amplified Fragment Length Polymorphism
Bp	Base Pair
BJC	Botswana Jatropha curcas
CTAB	Cetyl Trimethylammonium Bromide
CV	Coefficient of Variation
DAMD	Directed Amplification of Minisatellite DNA
DdH2O	Double – distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GJC	Ghana Jatropha curcas
IBPGR	International Board of Plant Genetic Resources
ISSR	Inter Simple Sequence Repeats
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PC	Principal Component

PCA	Principal Component Analysis
PIC	Polymorphism Information Content
PCOA	Principal Component Coordination Analysis
PCR	Polymerized Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SAHN	Sequential Agglomerative Hierarchical Nested
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
SIMQUAL	Similarity for Qualitative data
UPGMA	Unweighted pair group method with arithmetic averages
DAR	Department of Agricultural Research
RFLP	Restricted fragment length polymorphism
ITS	Internal transcribed spacer
NPGRC	National Plant Genetic Resources Centre

CHAPTER 1

INTRODUCTION

1.1. Origin and distribution of *Jatropha curcas*

The origin of *Jatropha curcas* remains controversial as it can be found over a wide range of regions in Central and South America in humid forest, tropical dry forest, cactus and thorn scrub, on shrubby slopes, thicket near river banks, dry steep hillsides, in woodland, hillsides with dense shrubs and woods, or coastal thickets (Heller,1996). According to Singh *et al.* (2010) *Jatropha curcas* originates from Central America and was spread to other tropical and subtropical countries. Its distribution was done by the Portuguese ships via the Cape Verde Islands and Guinea to other countries of Africa and Asia and nowadays, it is found in both tropical and subtropical countries all over the world (Henning, 2009).

The jatropha industry is still new in many parts of the world, covering a global area estimated at some 900 000 ha. More than 85 percent of *Jatropha curcas* plantings are in Asia, chiefly Myanmar, India, China and Indonesia (Brittaine and Lutaladio, 2010). Africa accounts for only 12 percent or approximately 120 000 ha, mostly in Madagascar, Zambia, Tanzania and Mozambique (Renner *et al.*, 2008). Latin America has approximately 20 000 ha of *Jatropha curcas* plantation, mostly in Brazil (Brittaine and Lutaladio, 2010).

The worldwide plantation of *Jatropha curcas* was projected to grow to 4.72 million ha by 2010 and 12.8 million ha by 2015 (Gexsi, 2008). Indonesia was expected to be the largest producer in

Asia with 5.2 million ha, followed by Brazil in Latin America with 1.3 million ha and lastly Ghana and Madagascar with 1.1 million ha each (Gexsi, 2008).

1.2. Botanical description of *Jatropha curcas*

The genus *Jatropha* is a morphologically diverse genus and belongs to tribe *Joannesieae* of *crotonoideae* in the Euphorbiaceae family and contains about 175 known species (Dehgan and Webster, 1979). To a few the species includes, *Jatropha curcas, Jatropha gossypiifolia Jatropha gladdulifera, Jatropha integerrima, Jatropha nana, Jatropha spicata Jatropha botswanica, Jatropha schlechteri* of which only four species have been recorded in Botswana namely; *Jatropha botswanica, Jatropha schlechteri and Jatropha. spicata, and Jatropha curcas.* Despite the diversity of the genus *Jatropha curcas* has received much attention because of its immense role in biodiesel production as renewable energy (Pandey *et al.,* 2012). *Jatropha curcas* is a diploid plant species with 22 chromosomes (Dehgan, 1984) with small genome size of about 416 Mb (Sato *et al.,* 2011). It is a perennial tree or shrub with woody succulent, smooth gray bark which discharges watery latex when cut. Generally, the tree can grow between 5.5 m and 9 m in height under favorable conditions (Nahar and Ozores-hampton, 2011). The leaves are cordate having about 1 to 7 shallow lobes and are arranged in alternate with spiral phyllotaxis.

The trees are deciduous with flowering occurring during the wet season and in permanently humid regions, throughout the year (Kamal *et al.*, 2011). The inflorescence is axillary paniculate polychasial cymes. Male and female flowers are produced on the same inflorescence, with an average of 20 male flowers to each female flower or 10 male flowers to each female flower (Kamal *et al.*, 2011). The plant is monoecious and flowers are unisexual; occasionally hermaphrodite flowers occur (Sunil *et al.*, 2010). A flower is formed terminally, individually,

with female flowers (tricarpellary, syncarpous with trilocular ovary) usually slightly larger and occurs in the hot seasons. The petiole length ranges between 6 - 23 mm (Kamal *et al.*, 2011). In condition where continuous growth occurs, an unbalance of pistillate or staminate flower production results in a higher number of female flowers. The ratio of male to female flowers ranges from 13:1 to 29:1 (Raju and Ezradanam, 2002) and it is decreases with tree age. The female flowers produce fruits that are first green, and turn yellow when ripening with the yellow fruit hull turning brown later and black when they dry. They remain fleshy until the seeds are mature after 2 - 4 months and finally separating into 3 cocci (Tiwari *et al.*, 2007). The size of fruit is about 2.5 - 3.5 cm long and 2 - 2.5 cm wide. The mature seeds are black, oblong, 2.5 to 3 cm long and 1 cm thick, more or less spherical with very small caruncles and are toxic to humans and animals (Henning, 2009).

1.3. Uses and importance of *Jatropha curcas*

Jatropha curcas is a multipurpose plant with many attributes of considerable potential in bioenergy systems (Openshaw, 2000). The seed of contain viscous, non-edible oil, which besides being source of bio-diesel can also be used for manufacturing other useful products such as candle, high quality soap, cosmetics and can be used for healing several skin disorders (Openshaw, 2000). The seed cake can be used as organic fertilizer and feedstock for biogas production (Staubmann *et al.*, 1997).

Beside the above uses other uses include soil erosion control and eco-restoration. The trees have proven effective in reducing the erosion of soil by rainwater. The lateral and adventitious roots near the surface bind the soil particles together preventing them from being washed out by raining water or heavy rains (Parawira, 2010). It also improves rainwater infiltration when

planted in lines to form contour bunds (Brittaine and Lutaladio, 2010). The hedges planted closed together form a barrier that is impenetrable to livestock and are used to protect the fields and gardens since animals do not browse it (Gubitz *et al.*, 1999).

1.4. Fatty acids composition of *Jatropha curcas* oil

Jatropha curcas seed oil contains both saturated fatty acids and unsaturated fatty acids as shown in Table 1 (Gubitz *et al.*, 1999) and they yield 25 – 40% oil by weight (Heller, 1996).

Table 1: Fatty Acids Composition in *Jatropha curcas* oil (Heller, 1996)

Name of fatty acid	Common name	Structure
Palmitic	Long chain fatty acid	C 16:0
Stearic	Long chain fatty acid	C 18:0
Oleic	Long chain unsaturated fatty acid	C 18:1
Linoleic	Long chain unsaturated fatty acid	C 18:2
Linolenic	Long chain unsaturated fatty acid	C 18:3
Mysteric	Medium chain saturated fatty acid	C 14:0
Palmitotic	Long chain fatty acid	C 16:0
Arachidic	Very long chain saturated fatty acid	C 20:0
Behenic	Very long chain saturated fatty acid	C 22:0
Cis – 11- eicosenoic	Very long chain unsaturated fatty acid	C 20:1
Cis- 11, 14 eicosadienoic	Very long chain unsaturated fatty acid	C 20:2

1.5. Climatic Requirements

Jatropha curcas is known for its ability to survive in very poor dry soils in conditions considered marginal for agriculture, and can even root into rock crevices (Brittaine and Lutaladio, 2010). However, survival ability does not mean that high productivity can be obtained from *Jatropha curcas* under marginal agricultural environments (Brittaine and Lutaladio, 2010). It grows well in tropical and sub-tropical regions, with cultivation limits at 30°N and 35°S. Also it can grow in temperate regions but performed very badly in terms of yield (Brittaine and Lutaladio, 2010). It is not sensitive to day length (flowering is independent of latitude) and may flower at any time of the year (Heller, 1996). It is a succulent shrub that shed its leaves during the dry season, with deep roots that make it well suited to semi-arid conditions. The optimum growing conditions are found in areas of 1000 to 1500 mm annual rainfall, with temperatures of 20°C to 28°C with no frost (Brittaine and Lutaladio, 2010). In areas with higher precipitation of about 3000 mm *Jatropha curcas* is likely to suffer from fungal diseases and restrict root growth (Foidl *et al.*, 1996) and high temperature causes reduction in yields. The plant thrives on different soil types, including infertile, gravelly, sandy, and/or saline soils (Dagar *et al.*, 2006). It can also thrive on the poorest stony soil with pH ranging from 5.5 to 9.0 (Foidl *et al.*, 1996) but it does best in aerated sands and loam soils of at least 45 cm depth. Heavy clay soils are less suitable and should be avoided, particularly where drainage is impaired, as *Jatropha curcas* is intolerant of waterlogged conditions (Brittaine and Lutaladio, 2010).

1.6. Statement of the problem and justification of the project

Jatropha curcas L. is undomesticated plant that has recently received great attention for its utilization in biofuel production, rehabilitation of wasteland, and rural development (Montes *et al.*, 201a). Botswana National Plant Genetic Resources Center (NPGRC) has conserved a vast number of *Jatropha curcas* germplasm. The available accessions lack genetic base information. Information on genetic diversity structure and marker-trait associations is urgently needed for the design of breeding strategies. The optimal design of a breeding strategy relies on the accurate information of the internal genetic structure and polymorphism of the germplasm at the breeder's hands. The optimal breeding category (clone, line, population, hybrid) for *Jatropha curcas* will then be defined in a comprehensive manner by combining knowledge of genetic structure, polymorphism, heterosis level and the cost to produce the improved cultivars. Morphological markers and isozymes have limited use in determination of genetic diversity and cannot adequately measure true genetic differences on their own (Jingura and Kamusoko, 2015) due to the strong influence of the environment on highly heritable seed traits like 100 seed weight, seed protein and oil content in *Jatropha curcas* (Heller,1996). However, DNA-based molecular markers provide a more efficient and powerful tool to study inter- and intra-specific genetic differences in jatropha (Jingura and Kamusoko, 2015). Therefore, application of more than one method is highly recommended for determination of intra-specific genetic diversity of *Jatropha curcas* in Botswana. The main goal of this study was to identify unique genotypes or sources of genetically diverse genotypes that will serve as parental line for *Jatropha curcas* breeding program in Botswana.

1.7. Objectives

1.7.1. General objective

1. To characterize accessions of *Jatropha curcas L.* grown in Botswana using morphological and molecular markers.

1.7.2. Specific objectives

1. To characterize accessions of *Jatropha curcas L.* grown in Botswana using morphological characters and Single Sequence Repeats (SSR).

2. To determine correlation between morphological markers and molecular markers in characterizing accessions of *Jatropha curcas L.* within Botswana.

1.8. Hypothesis of the research

H_O: There are morphological and molecular variations between *Jatropha curcas L.* accessions growing in Botswana.

H_A: There are no morphological and molecular variations between *Jatropha curcas L.* accessions growing in Botswana.

H_O: There is correlation between morphological and molecular markers in studied *Jatropha curcas L.* accessions.

H_A: There is no correlation between markers in studied accessions.

CHAPTER 2

LITERATURE REVIEW

2.1. Diversity evaluation using both morphological and molecular markers

Crop genetic diversity is important for crop adaptation to withstand pests and diseases and i provides the raw materials from which desirable or favorable alleles for improved agronomi traits of interest can be selected (Burow *et al.*, 2012). Various methods are available for use i estimating the genetic diversity of crops, such as morphological, biochemical and molecula markers. Measurements of genetic diversity can be generated using conserved accessions in gen banks (Parzies *et al.*, 2000). DNA-based molecular markers have several advantages over th conventional phenotypic markers since their presence is not dependent on the growth stage of th crop and can be found in all tissues (Mondini *et al.*, 2009).

2.2. Morphological markers

Morphological characterization is the first step in the classification and description of any cro germplasm (Smith and Smith, 1989). Morphological diversity is assessed by measuring variatio in phenotypic traits which have long been used in selecting crops that best suit needs of farme and also led to domestication of useful plants (Gepts, 2004). Evaluation of genetic diversit through morphological traits is direct, inexpensive and easy. However, morphologic estimations are more dependent on the environment and are more subjective than oth measurements (Ye *et al.*, 2009). Morphological variability depends on a limited number

genes, and may not access much of the potential variability for the agronomic traits present in a crop (Mayes *et al.*, 2009). Therefore, is important to use both morphological markers and molecular markers.

2.2.1. Genetic Diversity of *Jatropha curcas* using Morphological markers

Jatropha curcas is a polymorph species with variations reported in tree architecture, plant height, number of fruits, seed dimensions (length, width, lateral diameter) and seed weight (Guan *et al.*, 2013). In various studies, (Kaushik *et al.* 2007; Sunil *et al.* 2008; Saikia *et al.* 2015) some potentially important variations in *Jatropha curcas* trees have been detected. In a study conducted on 24 accessions of *Jatropha curcas* collected from different zones of India Kaushik *et al.* (2007) reported the variability in seed traits and oil content. There were significant differences ($P<0.05$) in seed size, 100 seed weight as well as in oil content between accessions. However, the coefficient of variation was higher for phenotype than genotype, indicating a predominant role of the environment. Similar results were found by Sunil *et al.* (2008a) selected promising accessions of *Jatropha curcas* from India, correlating morphological characteristics (plant height, collar height and thickness, number of primary branches, petiole length, number of fruits per cluster, pedicel length and seed yield) with the oil content of the seed.

Gohil and Pandya, (2008) studied fourteen characters in Indian *Jatropha curcas* accessions finding moderate genetic diversity and none of the morphological variables had heritability of over 75%. In another study of Indian accessions, Saikia *et al.* (2015) compared 34 sources, finding moderate variation in plant height, stem girth, branches per plant and seed weight. The studies conducted previously had indicated that there was morphological variation in *Jatropha curcas* germplasm growing in different parts of the world. The low cost, simplicity and

agricultural relevance of morphological characterization makes it an important tool in germplasm genetic variation studies. Therefore, results from the previous studies showed that morphological markers are still valuable technique to consider when characterising accessions of *Jatropha curcas* any way in the world.

2.3. Molecular Markers

Molecular markers are fixed marks in the genome found at specific locations of the genome. They are used to identify specific genetic differences (Semagn *et al.*, 2006). In order to precisely identify traits of interest, the marker must be close to the gene of interest so that the allele of both the marker and the gene could be inherited together. DNA markers are passed on from one generation to another through the laws of inheritance (Semagn *et al.*, 2006). Molecular markers are very stable, when compared to morphological characters, which may be influenced by environmental factors and having continuous variation and high plasticity (Weising *et al.*, 2005). The selection criteria could be based on the cost, technical labour, level of polymorphism, reproducibility, locus specificity and genomic abundance (Garcia *et al.*, 2004). Molecular markers are useful in the development of genetic and physical maps, and have increased the efficiency of indirect selection of marker linked traits. Generally markers are classified into hybridisation based DNA markers and PCR-based DNA markers (Gupta *et al.*, 1999). Based on the development of these techniques in the last three decades they are classified into three; the first generation molecular markers, including, restricted fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and their modifications; the second generation molecular markers, including simple sequence repeat (SSR), Amplified fragment length polymorphism (AFLP) and their modified forms and the third generation molecular markers including expressed sequence tag (EST) and single nucleotide polymorphism (SNP) (Gupta *et*

al., 2001). Molecular markers are being used as versatile tools for investigating various aspects of plant genomes (Jonah *et al.*, 2011). These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analyses of genome evolution, population genetics, taxonomy, plant breeding and diagnostics (Joshi *et al.*, 2011). In plant breeding molecular markers enhance the efficiency of selection of desirable traits via marker-assisted breeding and understanding the genetic relationships, evolutionary trends and fingerprinting of varieties of the same plant species (Jonah *et al.*, 2011).

2.3.1. Genetic Diversity of *Jatropha curcas* using Molecular Markers

1) DNA-based molecular markers are increasingly becoming important tools in plant genetic diversity analysis due to their sensitivity and specificity (Jingura and Kamusoko, 2015). The first advancement of DNA-based markers came with the introduction of Restriction Fragment Length Polymorphism (RFLP). Since then, the advent of Polymerase Chain Reaction (PCR) has provided new marker systems for diagnosis of genetic diversity to improve plant breeding programs (Jingura and Kamusoko, 2015). These are simple and quick techniques such as Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR), and Simple Sequence Repeat (SSR) or Microsatellites (Singh *et al.*, 2010). The use of DNA-based markers has precedence in genetic characterisation of *Jatropha curcas*. In India and China, a wide variety of molecular marker systems have been used to assess intra-specific genetic diversity within species of *Jatropha curcas* involving accessions from different agro-climatic zones (Singh *et al.*, 2010).

2.3.1.1. Simple Sequence Repeat markers (SSR)

Microsatellite markers, also known as SSR markers, consist of tandem repeats of short (one to six bp) nucleotide sequences (Hayden and Sharp, 2001). These are the stretches of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra- and penta- nucleotide units, which are arranged throughout the genomes of most eukaryotic species. For example, AAAAAAAAAAA would be referred to as (A)11, GTGTGTGTGTGT would be referred to as (GT)6 and ACTCACTCACTCACTC would be referred to as (ACTC)4 (Pokhriyal *et al.*, 2012). SSR markers are used widely because they are highly polymorphic, locus specific, abundant, spread over the genome and need just small amount of DNA for analysis (Yildirim *et al.*, 2009). These features make SSR an excellent molecular marker system for many types of genetic analysis, including linkage mapping, germplasm surveys and phylogenetic studies (Yildirim *et al.*, 2009). The polymorphism of SSR was first demonstrated in soybean (Akkaya *et al.*, 1992). Since then, the development of isolations of SSR and their application in genome analysis of many different plant species have accumulated rapidly (Rar and Akkaya, 2001). The development of SSRs markers, however, is costly and time consuming; therefore, it is important to test the utility of these SSR markers among related species (Yildirim *et al.*, 2009). According Castillo *et al.* (2010), homology exists among several related crop genomes in the sequences flanking the SSR loci and thus primer pairs developed for a species can often be used in related species. Owing to their abundance and inherent potential for variation, SSR (namely microsatellites) have become a valuable source of genetic markers in various aspects of molecular genetic studies.

Sun *et al.* (2008b) developed 17 genomic SSR, out of which only one was polymorphic among the 58 accessions of *Jatropha curcas* collected across China. Similarly, Cai *et al.* (2010) investigated the genetic diversity of 219 *Jatropha curcas* accessions from China using SSRs

markers and revealed a low genetic diversity in the Chinese germplasm. Pamidimarri *et al.* (2009a) isolated SSR markers and investigated the genetic diversity of *Jatropha curcas* accessions from India, and found a narrow genetic diversity in accessions. Also low polymorphism was found in 64 genotypes from five geographic locations (Brazil, Cape Verde, Cuba, Mozambique and Senegal) using 32 SSR markers (Ricci and Chekhovskiy, 2012). Ambrosi *et al.* (2010) analysed 26 accessions from different geographical regions (including Mexico, South America, Asia and Africa), using 10 RAPD, 6 ISSR and 10 SSR markers. Low genetic variability was documented not only among accession groups but also among accessions of different geographical origin, with the exception of Mexican landraces. Tanya *et al.* (2011) characterized 26 Mexican, 3 Chinese, 3 Thai and 4 Vietnamese accessions using SSR markers. Five of these loci clearly displayed distinct banding patterns between 26 Mexican accessions (non- toxic) and the 10 Asian accessions (toxic).

In a study undertaken by de Bressan *et al.* (2012), nine polymorphic microsatellite loci with 2–8 alleles per locus were identified, of which six loci showed transferability to three congeners: *Jatropha podagrica*, *Jatropha pohliana* and *Jatropha gossypiifolia*. Based on the whole genome sequences by Sato *et al.* (2011), they identified about 41000 simple sequence repeats loci in the 289 Mb sequences of the *Jatropha curcas* genome. From these, 100 SSR markers were developed and examined for polymorphism among 12 *Jatropha curcas* varieties obtained from Indonesia, Thailand, China, Mexico, Guatemala, Tanzania, Madagascar, Cape Verde and Uganda. The results showed that the polymorphism of those SSR markers in the limited accessions tested was low, but the accessions from Mesoamerican regions were genetically distinct from other regions.

Beside *Jatropha curcas* genetic diversity study, SSR markers have been used to assess the genetic diversity of various crop species (Singh *et al.*, 2008; Ribeiro *et al.*, 2011; Burow *et al.*, 2012). A study on assessment of genetic diversity among pigeonpea genotypes using 22 SSR markers revealed that the genotypes were highly diverse. Out of 22 SSR primers tested, 14 primers showed polymorphism and providing proper amplification pattern. A total of 46 amplified products were obtained with an average of 2.1 amplicons/ primers out of 32 being polymorphic bands. It was concluded that crossing among these genotype would likely produce highly heterotic individuals and could be used in heterosis breeding (Singh *et al.*, 2008).

On sorghum study by Burow *et al.* (2012) the genetic diversity of Chinese sorghum was moderate and 98% of SSR markers were found to be polymorphic with polymorphism information content value ranging from 0.05 to 0.92. The genetic variability among 93 Brazil cassava accessions based on 14 SSR markers was found to be very low (Ribeiro *et al.*, 2011). The coefficient of similarity between the 93 accessions ranged from 0.16 - 0.96.

The genetic diversity of *Jatropha curcas* has been assessed or analyzed in many parts of the world with other molecular markers beside the SSR marker. These markers revealed different degree levels of genetic diversity that ranged from low, moderate and high.

2.3.1.2. Single-nucleotide polymorphisms (SNPs)

Single-nucleotide polymorphisms are single-base pair positions in the genomes of two individuals at which different sequence alternatives exist in populations (Weising *et al.*, 2005). Single nucleotide polymorphism (SNP) markers have attracted significant attention in creating dense genetic linkage maps and genome-wide association studies (Wang *et al.*, 2011) because

SNPs are the most abundant class of polymorphisms in genomes, and can be genotyped cost-effectively (Rafalski, 2002). Single-nucleotide polymorphisms or SNPs are DNA sequence variations that occur when a single nucleotide (A, T, G and C) in the genome sequence is altered. SNPs constitute the most abundant of molecular markers. The high density and mutational stability of SNPs make them particularly useful DNA markers for population genetics and for mapping candidate genes for disease resistances and abiotic stress tolerance (Alves *et al.*, 2008). For example a SNP might change the DNA sequence AAGGCTAA to ATGGCTAA. The variation considered to be a SNP, if it occurs in at least 1% of the population and it can occur in both coding and non-coding regions of the genome (Coles *et al.*, 2005). SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. SNPs that are not in protein-coding regions may still affect gene splicing, transcription factor binding, or the sequence of non-coding RNA (Rafalski, 2002). SNP markers have emerged as an increasingly valuable marker system for assessing population genetic structure in different species in recent years.

Yang *et al.* (2012) sequenced three gene fragments, namely, ITS (internal transcribed spacer), PGIC (cytosolic phosphoglucose isomerase) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in 15 *Jatropha curcas* accessions, including three of its allies (*Jatropha gossypiifolia*, *Jatropha podagrica* and *Jatropha integerrima*). The results showed that within *Jatropha curcas* germplasm, nucleotide sequences were highly conserved, but the variation was high among inter-species. Silva-junior *et al.* (2011) reported on the discovery of a 768 high-quality SNPs for *Jatropha curcas* derived from a pool of genetically diverse accessions using Illumina sequencing and an SNP selection pipeline. These SNPs would facilitate further breeding and genetic improvement of *Jatropha curcas* in practice.

2.3.1.3. Amplified fragment length polymorphism (AFLP) analysis

AFLP markers consist of known sequence of 20 nucleotides and are called adapters (Vos *et al.*, 1995). The target DNA sequences are DNA fragments generated by restriction enzymes. The fragments are produced by genomic DNA by the combined action of two restriction enzymes. The adapters are ligated to each end of a restriction fragment by a protein ligase (Vos *et al.*, 1995). This technique is reliable, allows high throughput and is cost-effective. AFLP markers were used by a number of researchers (Sun *et al.* 2008; Tatikonda *et al.* 2009; Pamidimarri *et al.* 2009b; Shen *et al.* 2010; Zhang *et al.* 2011; Shen *et al.* 2012) for investigation of the genetic diversity in *Jatropha curcas* accessions from India, China, Brazil and Mexico. Various reports revealed contrasting results on the extent of genetic diversity of the crop. Pamidimarri *et al.* (2009b) reported low genetic variability among toxic *Jatropha curcas* accessions from India and wide variability between toxic Indian accessions and a non-toxic Mexican accession. In contrary, Tatikonda *et al.* (2009) reported a broad genetic base of *Jatropha curcas* in India. In addition Sun *et al.* 2008a; Shen *et al.* 2010; Zhang *et al.* 2011) reported lack of genetic variations in *Jatropha curcas* accessions in Asian and African countries. Shen *et al.* (2012) characterized the genetic variation among 63 populations of *Jatropha curcas* from 10 countries in Asia, Africa and Mexico and found that genetic diversity in Chinese populations were low, while the populations from Mexico displayed higher genetic diversity. Based on genetic diversity evaluation and analysis of molecular variance analysis, researchers explained the fact that *Jatropha curcas* germplasm from Mexico and Central America harbours greater genetic diversity than in other parts of the world (Ovando-medina *et al.*, 2011).

2.3.1.4. Randomly amplified polymorphic DNA (RAPD)

RAPD involves PCR amplification of genomic DNA using a single short oligonucleotide primer under low stringency conditions, which results in multiple amplification products from loci distributed throughout the genome. The technique is simple, rapid, inexpensive and applicable to any genome without any prior information regarding the genome of the plant (Subramanyam *et al.*, 2010). RAPD technique has been broadly applied in initial assessment of genetic diversity for *Jatropha curcas* in last 10 years. Basha and Sujatha, (2007) investigated the genetic diversity of 42 germplasm lines collected from different regions in India using RAPD and Inter Simple Sequence Repeat (ISSR) markers and the investigation revealed low inter-accessional variability. Kumar *et al.* (2009) as well measured the level of genetic diversity in 26 *Jatropha curcas* accessions collected from India. Their results indicated that 26 decamer primers produced 6011 amplification products, of which 30.9% were found to be polymorphic and the size of bands ranged from 300 to 2500 bp. Out of 43 RAPD primers, ten polymorphic primers (percentage in Brazil *Jatropha curcas* germplasm. In Africa, to exploit the *Jatropha curcas* germplasm for production of commercial bio-fuel in Kenya, Machua *et al.* (2011) determined the genetic diversity and genetic structure of 160 individuals collected from eight populations in Kenya using RAPD primers. Their results showed that the *Jatropha curcas* germplasm has a broad genetic base. In contrary, RAPD analysis of 40 accessions from Ghana with ten RAPD primers revealed an average polymorphism of 24.9%, indicating a narrow genetic base. In Asia the genetic diversity of *Jatropha curcas* accessions were conducted by several researchers in (Gupta *et al.*, 2008; Boora and Dhillon, 2010; Subramanyam *et al.*, 2010; Khurana-kaul *et al.*, 2012) and reported low to moderate level of genetic diversity in Indian accessions. In contrary, high level of genetic variation in Malaysian and Chinese *Jatropha curcas* accessions (Biabani *et al.*, 2012;

Rosado *et al.* (2010), performed a genetic diversity survey of 192 *Jatropha curcas* accessions collected from different geographical regions throughout Brazil using RAPD and simple sequence repeat (SSR) markers. Only 23 of the 381 RAPD markers were found to be polymorphic (6.2%) and the six SSR primers generated only eight different alleles in all the 192 germplasm accessions analysed, indicating a narrow genetic diversity and genetic improvement programmes.

2.3.1.5. Inter Simple Sequence Repeat (ISSR)

Inter-Simple Sequence Repeat (ISSR) amplification is another microsatellite-based technique useful for genome studies (Zietkiewicz *et al.,* 1994). Their amplifications are based on SSR sequences (di-, tri-, tetra-, or penta-nucleotide repeats),anchored to genomic sequences flanking each side of the targeted simple sequence repeats (SSR) (5' or 3') by using two to four arbitrary, and often degenerate, nucleotides (Monte-Corvo *et al.,* 2001). ISSR markers use arbitrary primers represented by short repeated sequences. Allelic polymorphisms occur whenever the repeated sequence is missing or insertions or deletions modify the distance between repeats (Intrieri *et al.,* 2010).

Technically simple, ISSR analysis has been successfully used in assessment of genetic variation in *Jatropha curcas* and genetic relatedness between *Jatropha* species from India, China and Brazil (Basha and Sujatha, 2007; He *et al.,* 2007; Xiang *et al.,* 2007; Basha *et al.,* 2009; Kumar *et al.,* 2011; Grativol *et al.,* 2011). Basha and Sujatha, (2007) characterized 42 *Jatropha curcas* accessions of native germplasm along with a non-toxic genotype from Mexico and reported moderate polymorphism (33.5%) with ISSR markers. Genetic diversity in six wild populations of *Jatropha curcas* collected from northeast India was assessed using ISSR and directed amplification of minisatellite DNA (DAMD) markers showed variation at intra-population level

was 68.9% (Kumar *et al.*, 2011). The genetic diversity of eight populations from China was estimated using ISSR primers, revealing a high level of genetic variation at species level (Xiang *et al.*, 2007). He *et al.* (2007) investigated the genetic diversity and genetic structure of nine populations in China and high level of genetic variation existed among the different populations. The genetic variability and genetic relationships of 332 *Jatropha curcas* cultivated accessions from 12 locations in Brazil were investigated using ISSR primers also showed high level genetic diversity at species level (Grativol *et al.*, 2011).

2.3.1.6. Expressed Sequence Tag (EST)-SSRs

EST is a DNA segment representing the sequence from a cDNA clone that is derived by reverse transcription from an mRNA molecule, or a part of it (Gupta *et al.*, 1999). EST-SSRs markers are those microsatellite loci derived from ESTs and have been applied to investigating genetic diversity of *Jatropha curcas* over the last 6 years. Malviya *et al.* (2011) mined SSRs from 13201 ESTs of *Jatropha curcas* and developed 21 EST-SSR markers for *Jatropha curcas*. Using 21 EST-SSRs, a total of 51 alleles in 25 accessions from India were detected with an average of 2.42 per primer pair. The polymorphism information content (PIC) value ranged from 0.04 to 0.61 with an average of 0.25, revealing low to moderate level of in formativeness with EST-SSRs in the *Jatropha curcas* accessions tested. Wen *et al.* (2010) studied the genetic relationships between 45 *Jatropha curcas* accessions from different countries using 36 EST-SSRs and 20 genomic-SSRs designs based on cassava sequence information. A total of 183 polymorphic alleles were detected, indicating that the *Jatropha curcas* germplasm tested in the study has a moderate level of genetic diversity. Regardless of the source of the germplasm that was subjected to characterization, genetic variation detected using SSR markers was rather low

in *Jatropha curcas*. Compared with AFLP, RAPD, ISSR makers, SSR (including EST-SSRs) markers exhibited lower genetic diversity in *Jatropha curcas* germplasm.

As per reviewed literature different molecular markers has proven to be robust technology for assessment of genetic diversity. These are used to assess questions of identity in order to identify putative duplicate accessions, and also to establish relationship and structure. Also to determines how variation is distributed among individuals. Therefore, it is important to consider use of molecular markers more specially the Simple Sequence repeats (SSR) in this study to characterise the genetic diversity of *Jatropha curcas* germplasm growing in Botswana.

CHAPTER 3

MATERIAL and METHODS

3.1. Experimental Site

The study was conducted at two sites; the molecular work was done at Botswana University of Agriculture and Natural Resources (BUAN) Tissue culture laboratory during period of October 2015 to May 2016. The morphological work was field based at Sebele Agricultural Research Station Nursery during the same period. The University is located along the A1 road about 10 km north of Gaborone City Center while the Agricultural Research Station Nursery is located at Sebele content farm which is about two kilometer Northwest of the BUAN, but about 2 km east of Sir Seretse Khama International Airport situated at S24°33.40 and E25°56.37 with an altitude of 992 m above the sea level. The nursery was established in 2011 with 86 *Jatropha curcas* accessions that were collected by National Plant Genetic Resources Center (NPGRC) from Southern and Northern parts of Botswana and 1 accession from Ghana (Figure 1). The nursery has a total of 771 *Jatropha curcas* trees planted from the indigenous accessions and 1 accession from Ghana. Each accession was planted in a single row with a maximum of ten trees spaced at 1.5 m and 2 m between rows. All the agronomic practices required for the establishment of healthy trees were undertaken and they irrigated twice a week during the dry period.

3.2. Plant Materials

A total of 30 *Jatropha curcas* accessions were used in the study and they are listed in Table 2 accompanied by their locality information. The accessions were selected based on their phenotype. It is only those that showed variations in their growth habit and also have more than three trees per accession. For data collection three trees per accession were randomly tagged. Thereafter morphological data and leave samples for molecular analysis were collected only from the tagged trees until the end of the study.

Table 2: List of *Jatropha curcas* accessions and their collecting sites

Accessions	Village	Region	Latitude	Longitude
GJC		Ghana		
BJC5	Mosetse	Northern	20°39.11	26°39.05
BJC7	Mosetse	Northern	20°39.19	25°39.20
BJC10	Tshesebe	Northern	20°45.13	27°35.34
BJC13	Moroka	Northern	20°32.13	27°38.59
BJC14	Nlapkwane	Northern	20°31.15	27°31.57
BJC17	Gabane	Southern	24°39.53	25°47.30
BJC18	Mogoditshane	Southern	24°56.44	25°50.27
BJC19	Metsimotlhabe	Southern	24°33.29	25°48.27
BJC20	Kumakwane	Southern	24°39.53	25°41.42
BJC22	Bokaa	Southern	24°43.28	26°02.08
BJC24	Molepolole	Southern	24°33.52	25°52.33
BJC25	Molepolole	Southern	24°33.52	25°52.33
BJC26	Tlokweng	Southern	24°39.19	25°58.30
BJC27	Tlokweng	Southern	24°39.19	25°58.30
BJC30	Gabane	Southern	24°39.53	25°47.30
BJC33	Kumakwane	Southern	24°39.08	26°10.14
BJC34	Kumakwane	Southern	24°39.01	25°41.42
BJC75	Moiyabana	Northern	22°37.50	26°24.22
BJC43	Maun	Northern	19°55.34	23°30.52
BJC45	Maun	Northern	19°55.34	23°30.52
BJC51	Letlhakane	Northern	21°24.49	25°34.21
BJC57	Mmatshumo	Northern	22°26.54	25°44.21
BJC62	Thabala	Northern	22°28.48	26°25.25
BJC65	Radisele	Northern	22°48.33	26°59.26
BJC66	Radisele	Northern	22°48.33	26°59.26
BJC67	Mahalapye	Northern	23°05.50	26°48.35
BJC68	Shoshong	Northern	23°02.17	26°30.36
BJC71	Mahalapye	Northern	23°05.50	26°48.35
BJC79	Serowe	Northern	22°26.56	26°44.48

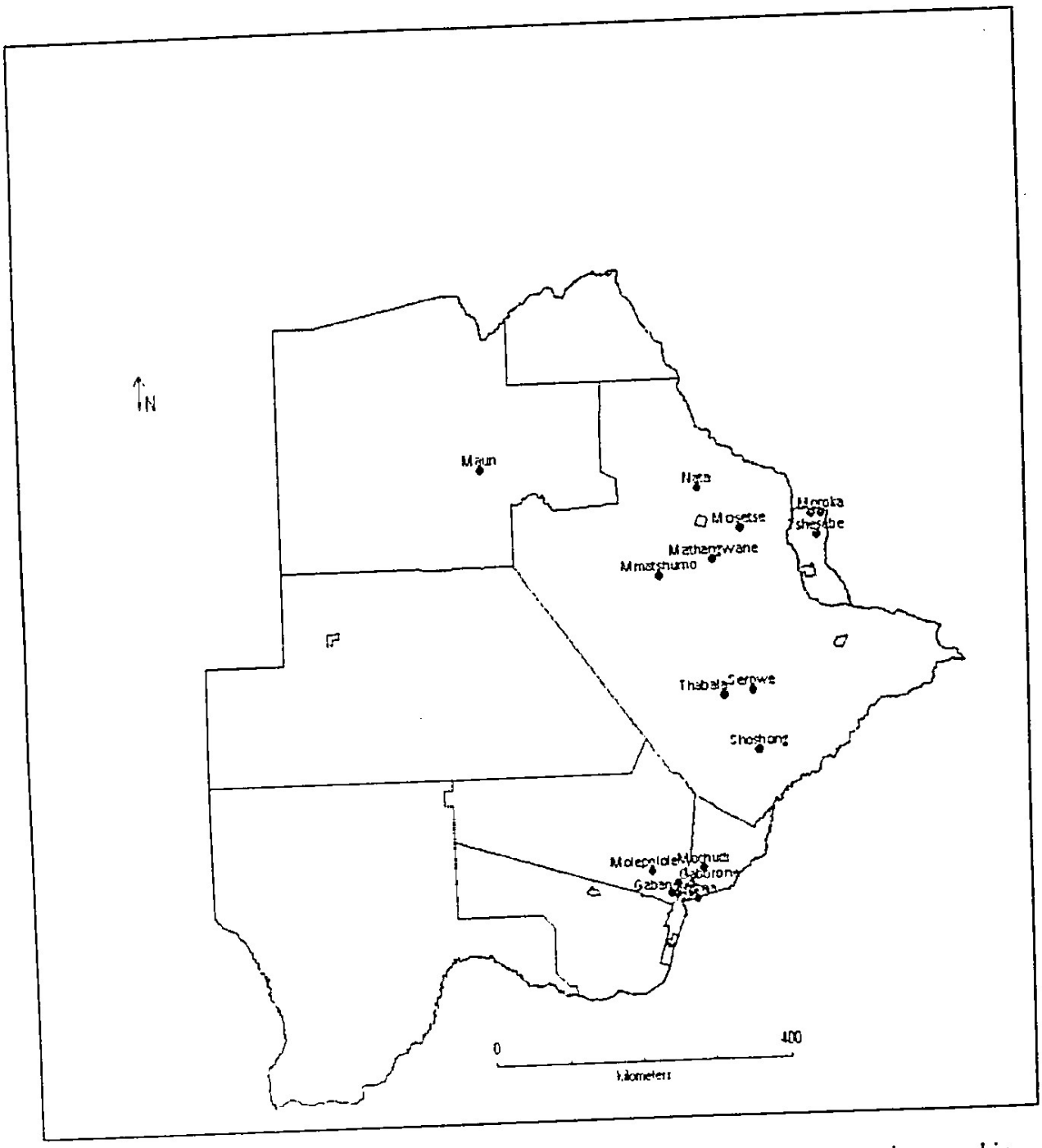

Figure 1: Map of Botswana showing the villages where the *Jatropha curcas* accessions used in the study were collected

3.3. Morphological data collection

A total of 24 of both qualitative (Table 3) and quantitative (Table 4) traits were recorded following the National Bureau of Plant Genetic Resources (NBPGR) minimal descriptor list for *Jatropha curcas* developed by Sunil *et al.* (2010). The generated morphological data were used to describe the morphology as well as morphological variability of *Jatropha curcas*.

Table 3: List of quantitative characters recorded and brief description as listed from NBPGR (Sunil, *et al.*, 2010)

Quantitative Characters	How the data was collected
1. Peduncle length	Peduncle was measured with ruler in millimeter (mm)
2. Number of female flower per cluster	The number of female flowers on fully expressed inflorescence were counted
3. Number of male flower per cluster	The number of male flowers on fully expressed inflorescence were counted
4. Length of fruit stalk	Physiologically matured fruits, measured with a ruler in millimeter (mm)
5. Number of fruits per cluster	Fruit per clusters were counted during peak podding stage
6. Number of seeds/fruit	Seed were counted on mature pods.
7. Fruit length (mm)	Measured on the physiologically mature fruits from 3 randomly selected plants with Vernier caliper
8. Fruit width (mm)	Measured on the physiologically mature fruits from 3 randomly selected plants with Vernier caliper
9. Seed length (mm)	Measured on the physiologically mature seeds from 3 randomly selected fruits with Vernier caliper
10. Seed width (mm)	Measured on the physiologically mature seeds from 3 randomly selected fruits with Vernier caliper
11. Seed weight (g)	Dried seeds were weighed with a balance in grams
12. Number of leaf lobes	Leaves lobes were counted on mature leaf of the current season growth on the tertiary branches.
13. Total flower per cluster	Counted before male drops
14. Leaf length	Measured on mature leaves on the tertiary branches with ruler
15. Leaf width	Measured on mature leaves on the tertiary branches with ruler
16. Petiole length	Measured on mature leaves on the tertiary branches with ruler

Table 4: List of qualitative characters and brief description as listed from descriptor list (Sunil *et al.*, 2010)

Qualitative Characters	How the data was collected	Descriptor
1. Flower color (FC)	Fully expressed inflorescence.	1.Cream yellow 2. White 3. Other
2. Inflorescence compactness (IFC)	Growth stage of the inflorescence as being depicted in	1. Loose 2. Semi-loose 3. Compact 4. Very compact.
3. Fruit shape (FTS)	Physiologically matured fruits.	1. Oval 2. Round
4. Petiole base pigmentation (PBP)	The terminal shoots of current season growth	1. Green 2. Purple 3. Brown
5. Pigmentation of emerging leaves (PEL)	Variability in pigmentation of young emerging leaves was recorded on the tertiary branches. Color was assigned as per the Royal Horticultural Society color chart	1. Green (Code: 142 A) 2. Green-greyed purple (Code: 140 A þ 185 D) 3. Yellow-green (Code: 149 A) 4. Greyed purple (Code: 185 A, B)
6. Stem color (SC)	Base or collar region of the main stem was considered for recording the stem color.	1. Green 2. Grey
7. Flowering occurrence (FO)	Recorded during the growing season	1.one flush 2.two flushes 3.continuous flushes
8. 6. Female: male flower ratio (F:MR)	The female and male flower ratio were recorded on the fully expressed Inflorescence	1. 1:10 2. 1:11-20 3. 1: > 20

3.3.1. Morphological Data Analysis

3.3.1.1. Quantitative and qualitative data descriptive characteristics

Data for all quantitative characters were subjected to analysis of variance (ANOVA) using the SAS version 9.3 General linearized models (GLM) package to determine the statistical differences on the traits for the given accessions. The mean values, ranges, standard deviation, coefficient of variation and F value were calculated on the 16 agro-morphological data. Qualitative data was summarized using Microsoft Excel.

3.3.1.2. Cluster Analysis

Cluster analysis, basically aims to find groupings in a set of individuals, objects or units such that individuals within a group are similar to each other but individuals in a different group are dissimilar to others. The data for each morphological character was first transformed using the STAND procedure in Numerical Taxonomy System-pc (NTSYSpc version 2.1 (Rohlf, 1998) in order to eliminate the effects of different scales of measurement. Then the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis was performed with NTSYSpc version 2.1 (Rohlf, 1998) using average linkage based on Euclidean distance and a dendrogram was produced to show the similarities and differences between *Jatropha curcas* accessions.

3.3.1.3. Principal Components Analysis

Principal component analysis (PCA) is a technique that summarizes patterns of correlations among observed variables and reduces a large number of observed variables to a smaller number of components with several linear combinations called principal components (Tabachnick and Fidel, 2007), with each principle component or eigenvalues being independent of other components. The importance of PCA is to extract maximum variance from the data set with each component. The new sets of transformed uncorrelated variables are close to the original variables but arranged in decreasing order of variance. PCA further enables plotting data in two dimensions to look at outliers, groups or clusters based on biological data (Chatfield and Collins, 1980). The quantitative and qualitative data were analysed using Principal Component Analysis in NTSYSpc, version 2.1 (Rohlf, 1998) based on the correlation matrix, which gives traits equal weightings.

Principal Component analysis (PCA) was used to reveal characters that account for most of variation between lines. The latent root or eigenvalue equal or greater than 1 were selected and used to define the agro-morphological diversity. Principal component analysis was constructed with NTSYSpc version 2.1 (Rohlf, 1998).

3.4. Molecular data collection

3.4.1. Sampling for DNA Isolation

The young leaves were collected from the tagged trees in the field and placed in absolute alcohol and transported to the laboratory. The DNA was extracted following the CTAB protocol as outlined below

3.4.1.1. DNA Extraction following the CTAB/Chloroform –Isoamyl Alcohol protocol

The leaf tissue was grounded in approximately 600 µL of CTAB buffer with automatic homogenizer. Then it was transferred to a 1.5ml tubes and 4 µL of RNASE was added and mixed by inverting the tube several times. The samples were incubated at 55°C for 1 hour in a heat block. Thereafter 500 µL of 24:1 Chloroform: Iso Amyl Alcohol was add and mixed well to form an emulsion by shaking the tubes with hands.

The samples were centrifuged for 10 minutes at 13 000 rpm speed and the aqueous phase of approximately 350 µL was pipetted into the new 1.5 ml tubes followed by 28 µL of cold 7.5 M ammonium acetate 204 µL cold isopropanol. The tubes were mixed well and left in the freezer for 1 hour. After 1 hour the tubes were centrifuged for 3 minutes at 13 000 rpm and the liquid was pipetted out leaving the DNA pellet at the bottom of the tube. The pellet was washed with 70% alcohol and the last wash done with absolute alcohol. The pellet was dried on hot plate at 55°C and later suspended with 100 µL TE buffer and stored at 4°C in a refrigerator.

3.4.1.2. DNA quality confirmation

The DNA purity was assessed with a Nanodrop 2000 Spectrophotometer (Thermo Scientific) at wavelength of 260 nm and 280 nm. Thereafter the genomic DNA was confirmed by loading the DNA samples in 1% agarose gel containing 2.5 µL of ethidium bromide staining and running the gel for 30 minutes at 100 volts. The gel was exposed to UV light to visualize the bands. The presence of a highly resolved high molecular weight band indicated good quality DNA. While the presence of smeared band indicated DNA degradation.

3.4.1.4. Polymerase chain reaction protocol

A total of twenty two (22) SSR primers pairs developed by Wang *et al.* (2011) (Table 5) were tested with 30 *Jatropha curcas* DNA sample for reproducible amplification. The PCR for the amplification of template DNA was performed in 2720 Thermo-cycler, Applied Bio system. The total volume of PCR reaction mixture was 10 µL, which contained, PCR buffer, dNTPs, ddH_2O, Ex-taq polymerase, DNA template and primer pairs. The PCR conditions for the microsatellite analysis were the touchdown PCR program as outlined in Table 6. The PCR products were separated on 3% agarose gel and stained with 2.5 µL of ethidium bromide. The gel documentation and visualization was done using the Bio-Rad Gel Doc systems XR + with image Lab Software.

Table 5: List of Simple Sequence Repeat primer (SSR) (Wang *et al.*, 2011)

Marker	Primer (5'-3', forward)	Primer (5'-3', reverse)	Annealing T°c
Jcuint018	TAGAAGAAAGCCCCGAAATG	GAGGGGATAAAAGAACGGTAAA	48.7
Jatr159	GATCAACTGCCAAGAGGACAAT	CTGATGAGCCCGTAACTGACA	51.5
Jatr691	ATCATTTTTGCCTCTTCTTTTGTC	GCCCACATGTGACCTTCCTAT	48.1
Jcuint143	GAATGCCCAAGAATAACCCACTT	CCACTTGGCCAGAAGGTAAGG	51.9
Jcuint048	CAAGATATGGGCAACAATCAATC	TACGCCAATTTATGGATACAGTGT	48.2
Jatr704	TGATGAATGTCCTCCAATAAACTT	GGAGCCCATATAGTAAACACGAG	47.8
Jcuint115	CTTATTTGTTGATGGAACCTCTTT	AACCCCCTTCTACATATGATAACA	47.0
Jcuint002	GATGTGGGATGCTGAGTGGTTTGA	ATTCTGAAGGCGGTGAGTGGTGAG	56.1
Jatr739	TTATGGAGGTTATTTAGAGGACAA	GGCCACCCCACTTTAGC	46.9
Jatr209	TAGTGCCCCCAGGTGATGAGTT	ACCTGCGTGTCCATTTGCTAC	54.5
Jatr915	ATCCACGCCATAGCTTTGTCACT	AATCGCTTATGGTGGCTGAAAAA	51.5
Jatr845	ATCTCACAACTGGGAACTAATGG	ATGCTCAGTCCACGGTAATCA	50.6
Jcuint060	TTTCCTTTGCATAAATTCATAGC	ATGCCAAAACTACCAAAATCAC	45.4
Jatr115	TGGGGTAGGCAAAATGTTAGAT	TTTTAGCGGGTTTTATTTTTAT	41.7
Jatr851	CAACCACTGAAAAAGACATACACA	TCGCTGACGCATGCTACTGAA	49.2
Jatr698	GAAATTATACACGAGCAAGTCTA	AAGGGGTGTAATTCCATCAG	46.0
Jcuint180	AAATGAGCCCTTGCAGGTCTATGC	AAACGCTACCGGACACTTGCTACA	56.6
Jcuint158	CAAAATTATGCAGGCTTGATGATA	AAAGGCACAACCAAGAATAAACAA	47.4
Jcuint020	CCACTGATGTAAATATGGACAGA	TAGCCCCACGCAATACCT	47.8
Jatr684	CCTTTCTTGGATATTTCGTCTCT	ACTTTGGGCCTGCTACTACCTC	48.3
Jatr117	TTGGCAGGCTTGTCTTAGTC	GACCCTTTTGCTTTGATTTTT	48.5
Jcuint079	AGGCAACATGGGTGTATCTTA	GAATCGTGCGGTCCTTCA	50

Table 6: Touchdown PCR 50/45 Program

Step	Temperature	Time
1. Initial Denaturalization	94°c	2 mm
2. Denaturalization	92°c	30 s
3. Annealing	50°c	30 s
4. Extension	72°c	30 s
5. Repeated 16 cycles of steps to 4		
6. Denaturalization	92°c	30 s
7. Annealing	45°c	30s
30 Extension	72°c	5 min
9. Repeated 18 cycles of step 6 to 8		
10. Final extension	72°c	5 min
Refrigeration	4°c	Indefinite

3.5. Molecular data analysis

3.5.1. Marker analysis

The markers were assessed for the in-formativeness and this was done by calculating polymorphism information content (PIC) for each marker. Thus:

$$PIC = 1 - \sum(pi)^2$$

Where, pi is the frequency of the *i*th allele in a population. PIC values take into account both the number of alleles at a locus and the relative frequencies of those alleles in a population under study. The markers loci with a large number of alleles occurring at equal frequencies will have the highest PIC values (Anderson *et al.*, 1993).

3.5.2. Cluster analysis

The amplified bands for simple sequence repeats (SSR) markers were scored as present (1) or absent (0) for each accession by manual inspection. The data were tabulated in a matrix using Microsoft Excel v2010. Data analysis was performed using Numerical Taxonomic and Multi-variant Analysis System (NTSYSpc version 2.1). The genetic similarity coefficient of pair-wise comparisons among the *Jatropha curcas* accessions analyzed were calculated based on Dice coefficient within the similarity for Qualitative data module of NTSYSpc version 2.1 (Rohlf, 1998). Clustering was performed using the generated coefficient according to the unweighted pair group mean algorithm (UPGMA) within the Sequential Agglomerative Hierarchical Nested analysis module. The dendrogram was drawn in the tree format using the tree plot module of NTSYSpc version 2.1 (Rohlf, 1998). The cophenetic correlation was estimated to measure goodness of fit among matrices generated using the matrix comparison module.

3.5.3. Principal component analysis

Principal Component Analysis (PCA) was used as one of the various techniques for the detection of possible relationships among *Jatropha curcas* accessions despite spatial distribution. PCA allows reduction of the variations exhibited to a manageable level and gives an easier interpretation and analysis of the data. The Principal Component Analysis (PCA) was performed

to determine the correlation on characters and the most significant traits contributing to variation in the accession through the generation of Eigen vectors and Eigen values. Also projection was done to compare the accessions and the projection file was combined with the Eigen vector (characters) file using the matrix plot option in NTSYSpc version 2.1 (Rohlf, 1998) to explain the character-based grouping of accessions.

CHAPTER 4

RESULTS

4.1. Characterization of *Jatropha curas* accessions using Morphological characters

4.1.1. Quantitative Characters

The coefficient of variation (CV) was used to compare the variability of different characters of 30 accessions. The characters that revealed higher variations are the number of female flowers per cluster (42.90%), number of male flowers per cluster (40.52%), peduncle length (39.55), total flowers per cluster (38.75%), length of fruit stalk (35.40%) and number of fruit per cluster (33.00%) (Table7).

Lower coefficients of variation were revealed in number of leaf lobes (9.13%), seed length (11.91%) and number of seed per fruit (13.00%) which indicates the small variation for these traits, and also suggesting that these are more likely to be highly heritable traits.

Highly significant differences ($P<0.001$) were detected among genotypes in 15 characters that were analyzed, except for number of leaf lobes (Table 7). This is an indication of substantial amount of genetic variability among the traits analyzed for this set of accessions.

Table 7: Mean Standard deviation, Range, Coefficient of variation, F-Value and P-Value of 30 *Jatropha curcas* accessions assessed based on 16 quantitative traits

Character	Mean	Std. dev	Range	CV%	F-Value	Pr>F
1. Peduncle length (PDL)	29.88	13.78	5-65	39.55	4.10	<0.0001
2. Number of seed/fruit (NSPF)	2.60	0.26	1-3.00	13.00	2.26	0.0036
3. Length of fruit stalk (LFS)	17.95	6.04	5-30	35.40	4.48	<.0001
4. Fruit length (FL)	20.16	3.52	15-35	17.80	9.46	<.0001
5. Fruit width (FW)	18.34	2.73	13-25	15.73	6.64	<.0001
6. Seed length (SL)	14.49	1.73	10-20	11.91	13.92	<.0001
7. Seed width (SW)	9.91	1.78	8-19	17.13	1.90	0.0003
8. Seed weight (SWHT)	1.41	0.36	0.61-2.05	25.53	1017.7	<.0001
9. Leaf width (LW)	85.14	11.35	65-130	14.46	9.17	<.0001
10. Leaf length (LL)	79.24	13.09	71-140	15.48	12.54	<0.001
11. Number of leaf lobes (NLL)	1.22	0.18	1-2	9.13	1.01	0.4705
12. Petiole length (PL)	90.17	14.74	65-140	16.55	7.15	<.0001
13. Total flowers/ cluster (TFC)	51.08	20.53	30-109	38.75	4.46	<.0001
14. Number of female flowers / cluster (NFFC)	2.95	1.30	1-7	42.90	2.35	0.0024
15. Number of male flowers /cluster (NMFC)	48.18	20.21	26-103	40.52	4.19	<.0001
16. Number of fruits per cluster (NFrPC)	2.62	0.40	1-2	33.00	3.80	0.0001

4.1.2. Distribution of qualitative characters amongst the accessions

The distribution of qualitative characters among the 30 accessions of *Jatropha curcas* studied was as follows; 90.3% displayed brown pigmentation at the base of the petiole while only 9.7% displayed green petiole base. Inflorescence compactness was distributed as 77.4% compacted and 22.6% loose compact while the flowering occurrence was distributed as 51.6% flowered twice, 38.7% flowered once and only 9.7% had continuous flowering. The proportion of female flowers to male flower was distributed as 38.7% (1:1-20), 35.5% (1:>20) and 25.8% (<1:10). The pigmentation of emerging leaves was distributed as 77% dark greyed purple, 22% greyed purple and 6.4% red. The grey stem color, cream yellow flower color and oval fruit shape, appeared in all the accessions and this was excluded for multivariate analysis (Table 8).

Table 8: Description of qualitative characters adopted and frequency of occurrence of the categories in the 30 accessions of *Jatropha curcas* (Sunil *et al.*, 2010).

Description	Categories	Relative frequency (%)
1. Stem color	Grey	100
	Green	0
2. Flower color	Cream yellow	100
	White	0
	Other	0
3. Fruit shape	Oval	100
	Round	0
4. Pigmentation of emerging leaves	Green (code 142A)	0
	Green greyed purple (code 140a+185D	0
	Yellow green (code 149A)	0
	Greyed purple (code 185A,B)	22.6
	Dark greyed purple (code 187A)	71
	Red (code 44)	6.4
5. Petiole base pigmentation	Green	9.7
	Purple	0
	Brown	90.3
6. Inflorescence compactness	Loose	0
	Semi –loose	22.6
	Compact	77.4
	Very compact	0
7. Female : Male flower ratio	1.<1:10	25.8
	2.1:11-20	38.7
	3.1:>20	35.5
8. Flowering occurrence	one flush	38.7
	two flushes	51.6
	continuous flushes	9.7

4.1.3. Morphological Multivariate Cluster Analysis

Morphological cluster analysis was performed based on 15 quantitative (Table 3) and 5 qualitative (Table 4) characters of 30 accessions. Four clusters (Figure 2) were produced at dissimilarity coefficient of approximately 1.37. The major clusters observed were labeled alphabetical A, B, C, and D. The first cluster (A) comprised of one accession which comes from Ghana (GJC1). The accession was separated from the rest of the accessions by seed weight and inflorescence compactness (Table 9).

Cluster B is the biggest with 23 accessions (BJC5, BJC7, BJC13, BJC18, BJC14, BJC20, BJC51, BJC43, BJC65, BJC67, BJC57, BJC26, BJC33, BJC34, BJC17, BJC22, BJC10, BJC24, BJC25, BJC62, BJC19, BJC30 and BJC45). These accessions were collected from different villages of Botswana. The major characters that led to the grouping were fruit stalk length, pigmentation on the emerging leaves, fruit length, fruit width, female to male flower ratio, pigmentation of peduncle base and number of seed per fruit. Within these cluster accession BJC14 and BJC20 were the related with the distance coefficient of about 0.51.

Cluster C is the second largest with five accessions (BJC66, BJC71, BJC68, BJC75 and BJC79) grouped according to their collecting sites. The major characters that led to the grouping were female to male flower ratio, number of seeds per fruit, number male flower per cluster and total flower per cluster.

Cluster D is comprises of one accession (BJC27) from Tlokweng village in the southern region and it was separated from the rest by having big leaves and very long petiole length (Table 9).

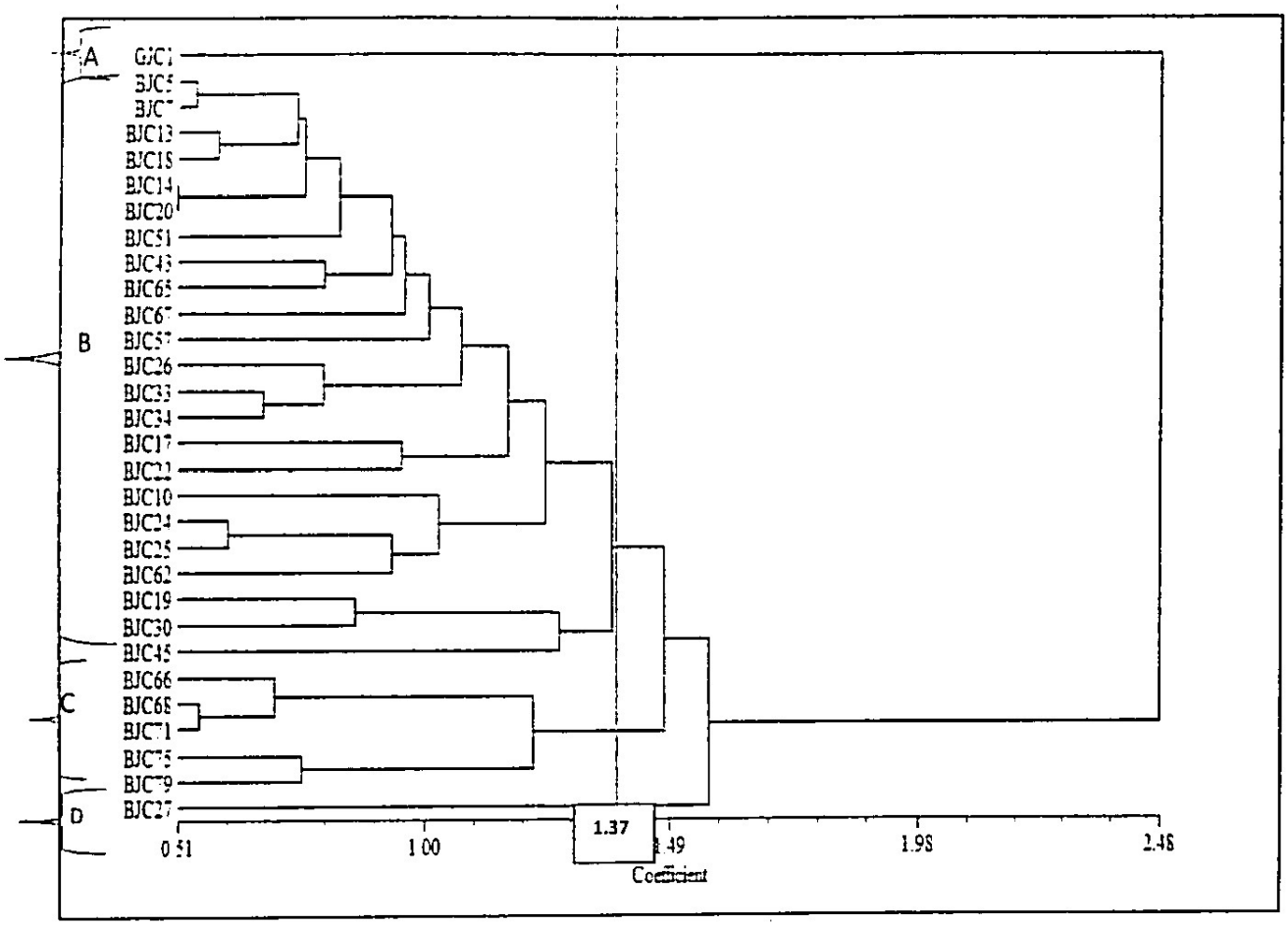

Figure 2: Hierarchical clustering using the distance coefficient. The y-axis shows the 30 accessions and the four clusters; X-axis indicates the distance coefficient between clusters. 4 clusters at coefficient approximately 1.37 are shown. The test of association: Matrix correlation on NTSYS pc (r=0.95).

4.1.4. Morphological Principal Coordination Analysis

The Principal Coordination Analysis (PCoA) revealed exactly the same four groupings obtained by cluster Analysis (Figure 3). The clusters were also designated by letter A, B, C and D. Cluster B being the biggest with 23 members followed by C with five accessions and lastly A and D comprising of only one accession each. The PCoA clearly separated GJC1 from the rest of the accessions.

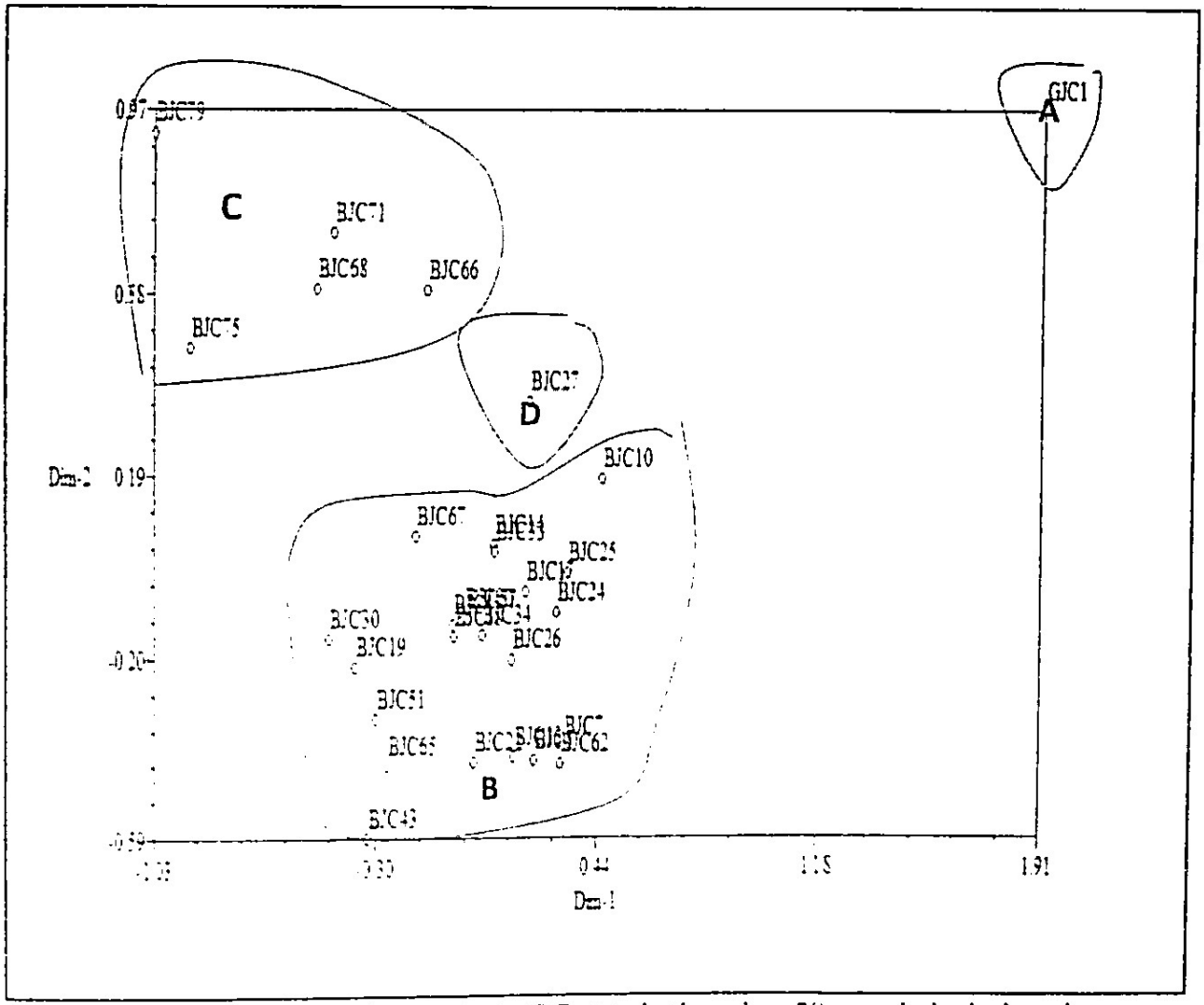

Figure 3: Principal Coordination Analysis (PCoA) plot based on 20 morphological markers revealing four major clusters

Table 8: Distribution of 30 *Jatropha curcas* accessions in four clusters based on their 20 morphological characters.

Cluster	Number of Accessions	Cluster identification	Cluster members
A	1	Seed weight and Inflorescence compactness	GJC
B	23	Fruit stalk length, pigmentation on emerging leaves, fruit length, fruit width, female to male flower ratio, pigmentation of peduncle base and number of seed per fruit	BJC5, BJC7, BJC13, BJC18, BJC14, BJC20, BJC51, BJC43, BJC65, BJC67, BJC57, BJC26, BJC33, BJC34, BJC17, BJC22, BJC10, BJC24, BJC25, BJC62, BJC19, BJC30 and BJC45
C	5	Female : male flower ratio, number of seeds per fruit, number male flower per cluster and total flower per cluster	BJC66, BJC68, BJC71, BJC75, and BJC79
D	1	Leaf length, leaf width and petiole length	BJC27

4.1.5. Morphological Principal Component Analysis

The 20 characters assessed were subjected to principal component analysis to identify characters that accounted for most of the variance in the 30 accessions. Only the first five principal components with eigenvalues more than one were selected, giving an accumulated total variation of 71.74% (Table 10). The first principal component (PC1) with the eigenvalue of 5.13 contributed 25.65% of total variation and characters with higher loadings were from leaf width (0.8211), seed length (0.8176), leaf length (0.7725), fruit width (0.7723), length of the petiole (0.7610), fruit length (0.7460), number of female flower per cluster (0.5837), seed width (0.5494), number of fruits per cluster (0.5132) which had maximum and positive weight on the this component. The female flower ratio: male flower ratio had negative weight (-0.4131) on PC1. The other traits like inflorescence compactness (0.1628), total flower per cluster (0.1329) had comparatively less effect on the first PC1.

The second principal component (PC2) with the eigenvalue of 3.49 accounted for 17.46% of the total variation and higher positive loadings were observed from mostly the inflorescence characters total flowers per cluster (0.9262), number of male flowers per cluster (0.9243), and female flower ratio to male flower ratio (0.5965). Negative loadings were observed for pigmentation on emerging leaves (-0.5214), length of fruit stalk (-0.5045) and seed width (-0.4476).

The third principal component (PC3) with the eigenvalue of 2.50 explained 12.51% of total variation and was mainly due to the positive loading from flowering occurrence (0.7105), length of fruit stalk (0.5710) and number of fruits per cluster (0.4673) as well as negative loading from seed weight (-0.5466) and number of seed per fruit (-0.4368).

The forth principal component (PC4) with eigenvalue of 1.87 explained 9.39% of the total variation and was due to the positive loading from female: male flower ratio (0.6088), and number of female flowers per cluster (0.4850). Negative loadings were observed for number of seed per fruit (-0.6023) and fruit length (-0.4458).

The fifth principal component (PC5) with eigenvalue of 1.35 explained 6.73% of the total variation. This was due to the positive loading from petiole length (0.4503) and leaf length (0.4330) as well as the negative loading from the peduncle length (-0.4855), seed weight (-0.4774) and number of fruit per cluster (-0.4473). This indicates the importance of these characters in identifying *Jatropha curcas* accessions.

Table 9: Principal component analysis of *Jatropha curcas* morphological traits showing proportion of variation associated with the first five components and eigenvector of traits

Characters	PC1	PC2	PC3	PC4	PC5
1. Peduncle length (PDL)	0.3975	-0.0359	-0.0470	-0.1092	**-0.4855***
2. Length of fruit stalk (LFS)	0.0985	**-0.5045***	**0.5710***	0.3921	0.3236
3. Number of seeds per fruit (NSPF)	-0.0300	0.3217	**-0.4368***	**-0.6023***	-0.0466
4. Number of male flowers per cluster (NMFPC)	0.0927	**0.9243***	-0.0141	-0.2662	-0.1270
5. Number of female flowers per cluster (NFFpc)	**0.5837***	0.1358	0.3822	**0.4850 ***	-0.2650
6. Total flower per cluster (TFpc)	0.1329	**0.9262***	0.0443	-0.2348	-0.1473
7. Number of fruits per cluster (NFrpc)	**0.5132***	0.2362	**0.4673 ***	0.1573	**-0.4473**
8. Leaf length (LL)	**0.7725***	0.3627	-0.1128	0.1102	**0.4330 ***
9. Leaf width (LW)	**0.8211***	0.3730	-0.0570	0.1327	0.3457
10. Petiole length (PL)	**0.7610***	0.2083	-0.2286	0.1477	**0.4503***
11. Fruit length (FL)	**0.7460***	-0.0945	-0.0293	**-0.4458 ***	0.0809
12. Fruit width (FW)	**0.7723***	-0.1684	0.0053	-0.0798	-0.2028
13. Seed length (SL)	**0.8176***	-0.2555	-0.2527	-0.1317	-0.0851
14. Seed width (SW)	**0.5494***	**-0.4476***	-0.3068	-0.2212	-0.0553
15. Seed weight (SWHT)	0.2630	0.1679	**-0.5466***	-0.2371	**-0.4774***
16. Inflorescence compactness (IFC)	0.1628	0.1913	0.3360	-0.1113	0.2372
17. Flowering occurrence (FO)	0.3449	0.1534	**0.7105***	-0.0205	-0.1622
18. Female :Male flower ratio (F:MR)	**-0.4131***	**0.5965 ***	-0.0582	**0.6088***	0.0519
19. Pigmentation on emerging leaves (PEL)	0.2004	**-0.5214***	0.2118	-0.0030	-0.2194
20. Pigmentation on petiole base (PPB)	0.2313	0.1067	0.3612	0.0636	0.1372
Eigen Value	5.13	3.49	2.50	1.87	1.35
Percentage	25.65	17.46	12.51	9.39	6.73
Cumulative percentage	25.65	43.11	55.62	65.02	71.74

* bolded face indicate relevant characteristic when explaining the component

4.2. Molecular characterization of *Jatropha curas* accessions using Simple Sequence Repeat (SSR) markers

A total of 30 *Jatropha curcas* accessions were analyzed using 18 SSR markers. The accessions were collected from different regions of Botswana and one accession was collected from Ghana and labeled Ghana *Jatropha curcas* (GJC). All the accessions collected from Botswana were labeled in numerals with the prefix Botswana *Jatropha curcas* (BJC). The identification of DNA fragments amplified by microsatellite markers was done by performing a visual analysis. Each marker band was evaluated for the 30 accessions, identifying the presence of a band by 1 and for an absence by 0 (Figure 4-7). The primers that amplified more than 50% of the samples were chosen for analysis.

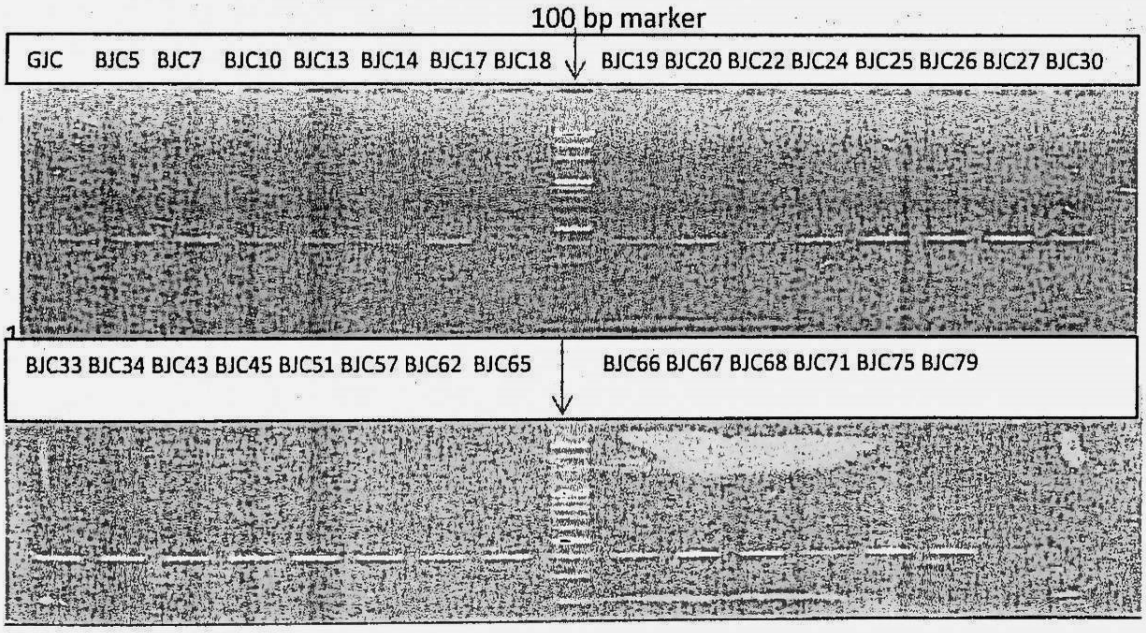

Figure 4: Amplification profile of 30 accessions of *Jatropha curcas* with Jatr115 primer

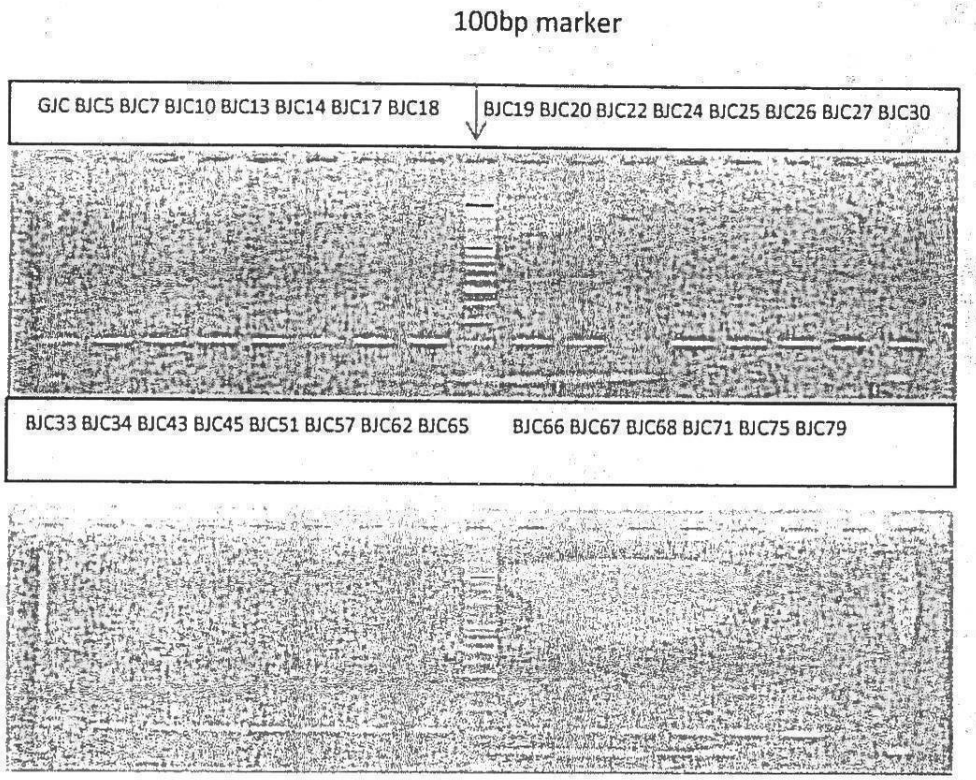

Figure 5: Amplification profile of 30 accessions of *Jatropha curcas* with Jatr159 primer

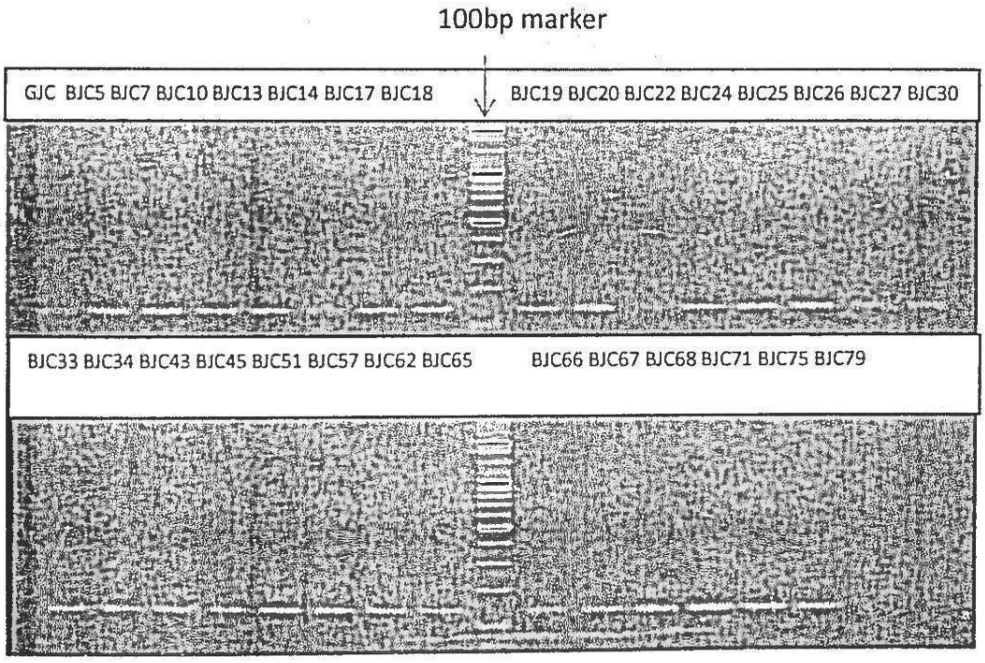

Figure 6: Amplification profile of 30 accessions of *Jatropha curcas* with Jatr691 primer

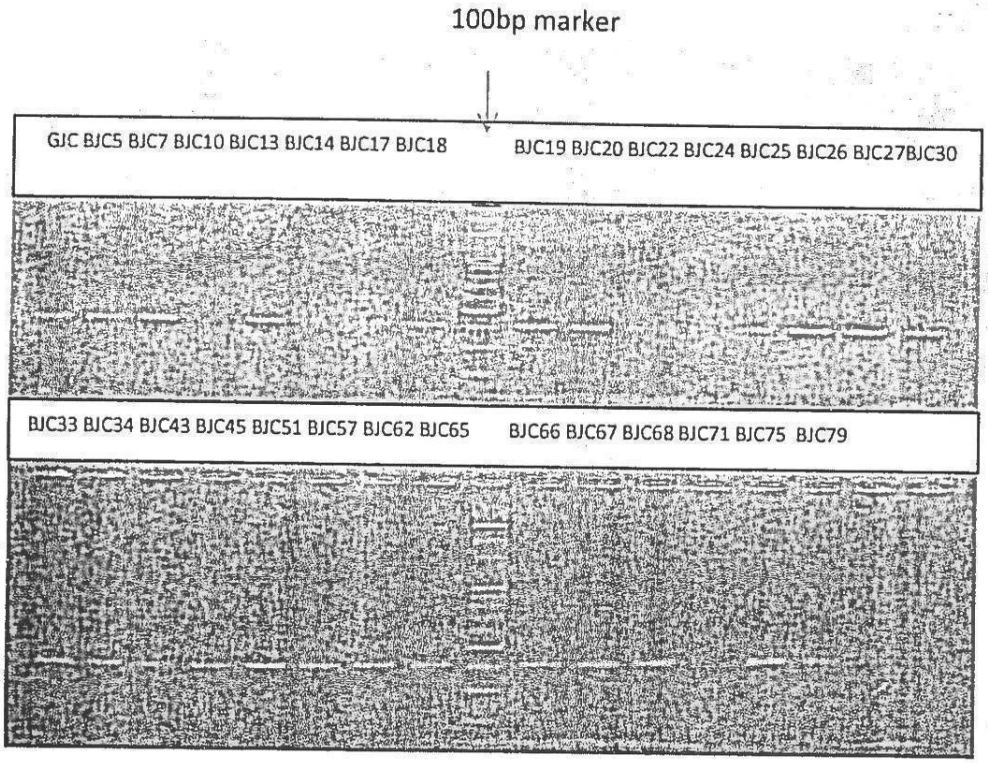

Figure 7: Amplification profile of 30 accessions of *Jatropha curcas* with Jatr704 primer

4.2.1. Microsatellites marker analysis

The markers were assessed for polymorphism by calculating the polymorphic information content (PIC). The calculated PIC for each marker was 0.00 indicating no informativeness within SSR markers (Table 11).

Table 10: SSR primer pairs and their number of amplified bands, monomorphic bands and polymorphic bands for diversity study of *Jatropha curcas* accessions

SSR primer pair	Amplified Bands	No. of polymorphic bands	No. of monomorphic bands	PIC
Jatr704	26	0	26	0
Jcuint115	15	0	15	0
Jcuint002	25	0	25	0
Jatr739	21	0	21	0
Jcuint018	27	0	27	0
Jatr159	28	0	28	0
Jatr691	28	0	28	0
Jcuint143	28	0	28	0
Jatr851	29	0	29	0
Jatr698	28	0	28	0
Jcuint180	28	0	28	0
Jcuint158	29	0	29	0
Jatr209	27	0	27	0
Jatr845	29	0	29	0
Jcuint060	26	0	26	0
Jatr115	30	0	30	0
Jcuint020	25	0	25	0
Jatr684	25	0	25	0

4.2.1. Multivariate Molecular Cluster Analysis

The genetic relationships among the 30 *Jatropha curcas* accessions are shown in Figure 8. The amplitude in the similarity coefficient ranged from 0.71 to 1.00. The dendrogram obtained revealed four distinct cluster at 0.85 similarity coefficient, and grouping is from right to the left. The first cluster (A) comprised of one accession (GJC) and this accession came from Ghana. The second cluster (B) comprised of twenty seven accessions (BJC5, BJC7,BJC68, BJC17, BJC79, BJC19, BJC20, BJC25, BJC27, BJC34, BJC45, BJC51, BJC57, BJC62, BJC24, BJC65, BJC33, BJC26, BJC71, BJC43, BJC67, BJC18, BJC30, BJC75, BJC66, BJC10 and BJC13) which are all Botswana accessions but collected from different villages. These accessions were grouped regardless of their collecting sites. Eleven accessions which are BJC17, BJC79, BJC19, BJC20, BJC25, BJC27, BJC34, BJC45, BJC51, BJC57 and BJC62 had the highest similarity coefficient of 1.00 that is the used markers failed to distinguish them from one another and treated them as duplicates.

The third cluster (C) comprised of one accession (BJC14) which came from Nlapkwane village in the northern region and the forth cluster (D) comprised also of one accession (BJC22) which came from Bokaa village in the Southern region.

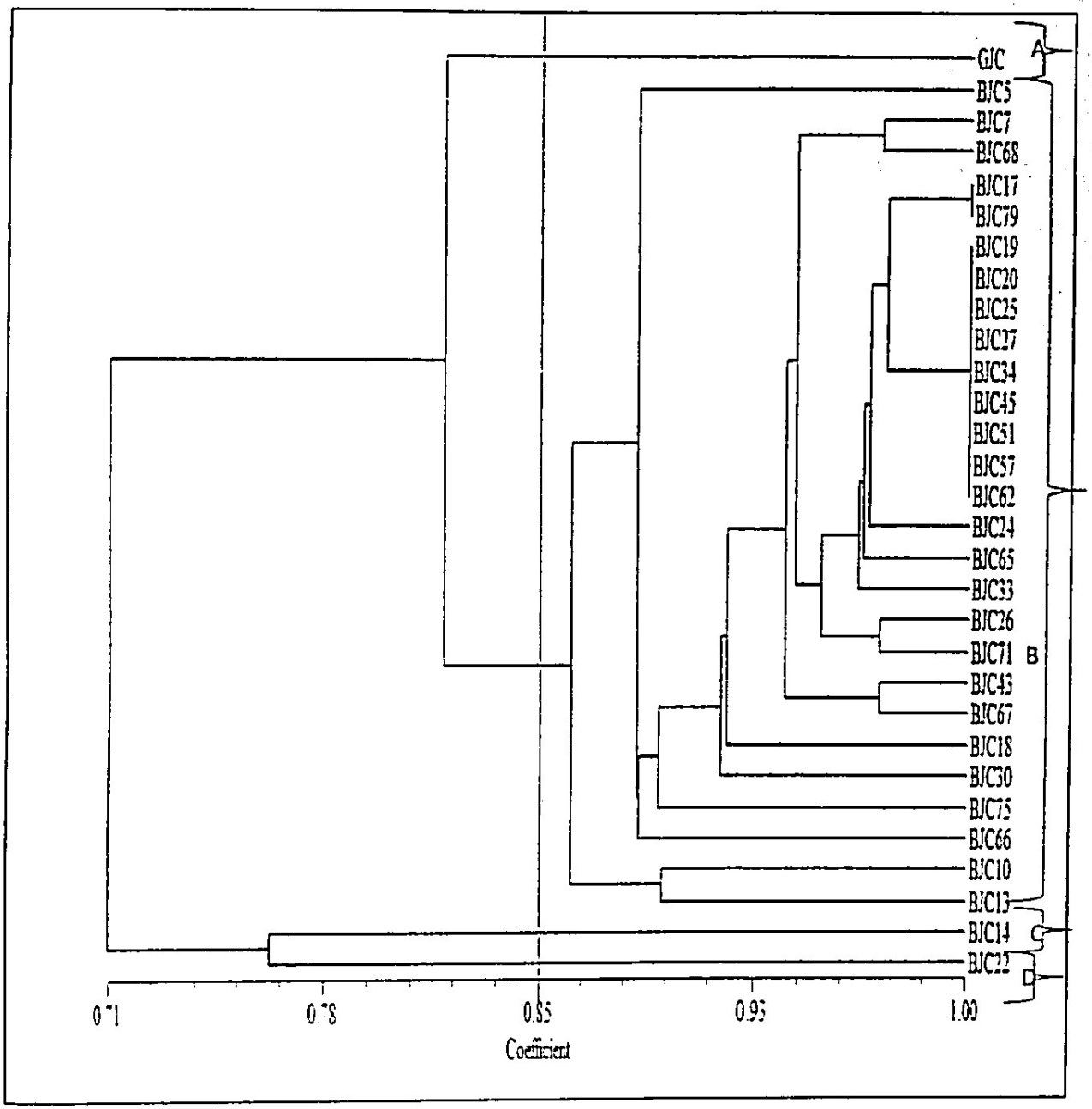

Figure 8: Hierarchical clustering using the similarity coefficient. The y-axis shows the 30 accessions and four clusters; x-axis indicates the similarity coefficient between clusters. Four clusters at coefficient approximately 0.85 are shown. The test of association: Matrix correlation on NTSYS pc (r = 0. 93).

4.2.3. Principal Coordination Analysis

The Principal Coordination Analysis (PCoA) revealed exactly the same four groupings obtained by cluster analysis (Figure 8). The clusters were also designated by letter A, B, C and D (Figure 9). Cluster B is the biggest with 27 members followed while cluster A, C and D are the smallest with one member each.

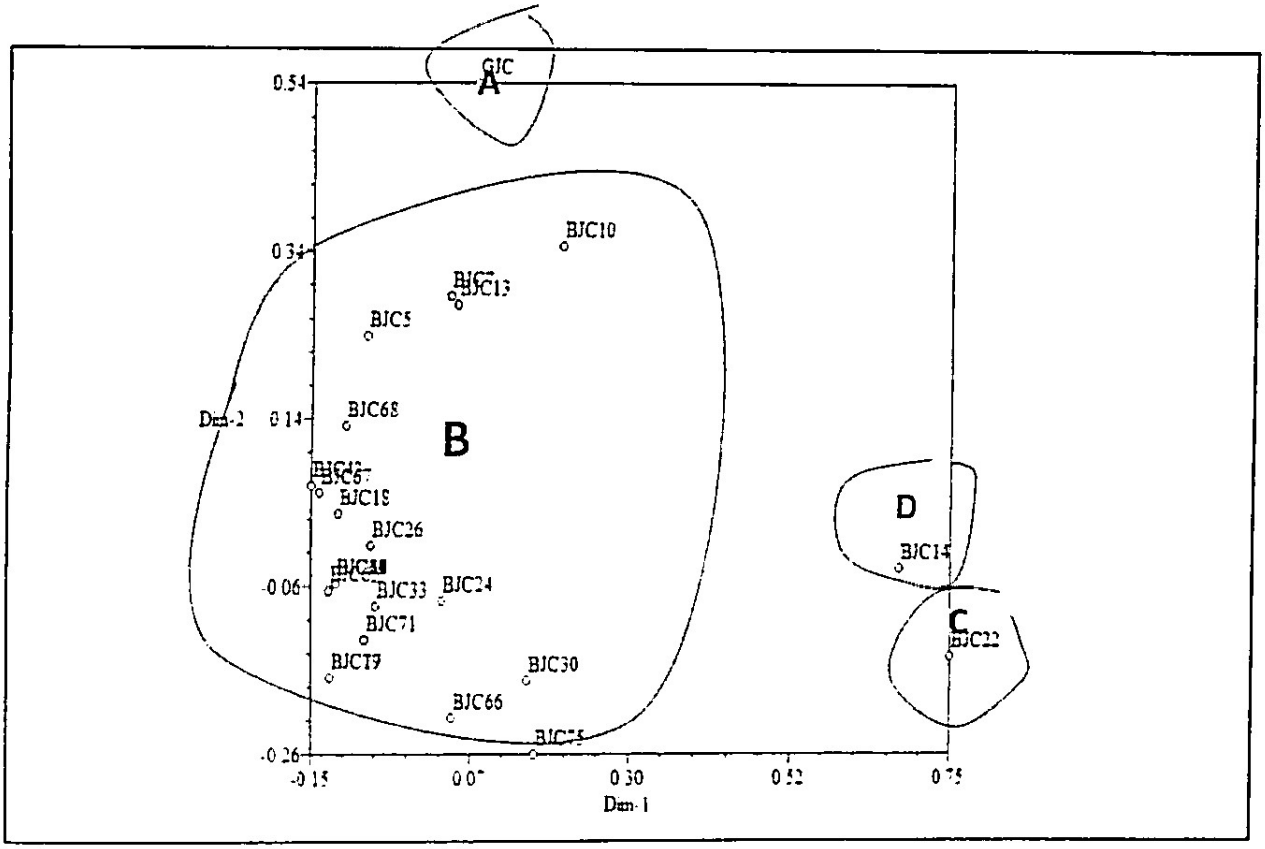

Figure 9: Principal Coordination Analysis (PCoA) plot based on 18 SSR markers revealing four clusters

4.2.4. Multivariate Principal Component Analysis

The Principal Component Analysis (PCA) was used as one of the various techniques for the detection of possible relationships among *Jatropha curcas* accessions despite spatial distribution. PCA allows reduction of the variations exhibited to a manageable level and gives an easier interpretation and analysis of the data. The accurate interpretations of the four principal components are made possible by the examination of the different eigenvector values which are the relative weights accounted for by the evaluated markers, that is with more than two eigenvalue or latent root. The sign (positive or negative) tells the direction that a given variable in that PC is going on a single dimension vector. The negative sign indicates: inverse relationship between factor and variable but magnitude tells the strength of relationship.

The PCA reduced the original 18 Simple Sequence Repeat markers into four principal components which accounted for 73.91% of the total cumulative variance (Table 12).

The first principal component (PC1) was the most important component, with eigenvalue of 4.76 explaining 26.45% of the total variation. This was due to the contribution of five markers Jatr691 (-0.9841), Jat209 (-0.9841), Jatr115, Jcuint158 and Jat845 loading -0.9701 each. All the five markers were loading in the negative direction. These five markers did not play any role in explaining the variation on the entire PCs (2, 3 and 4) and this was indicated by having a value of 0 for those PCs.

The second principal component (PC2) with eigenvalue of 3.56 accounted for 19.83% of the total variation. This was due positive loading from four markers which are Jcuint180 being the most loading with 0.9722 followed by Jcuint018 and Jcuint002 loading the same weights of

about 0.9540 each and lastly Jatr698 with 0.8967. Also, they did not play any role in explaining the principal components 1, 3 and 4.

The third principal component (PC3) with eigenvalue of 2.55 accounted for 14.19% of the total variation. The markers associated with PC3 were Jcu143 loading 0.9402, followed by Jcu048, and Jcu060 loading 0.9143 each in the positive direction and did not play any role in explaining other principal components.

The forth principal component (PC4) with the latent root of 2.42 contributed 13.45%. This was due to the positive loadings from Jatr704 (0.9169), Jatr739 (0.8894) and the negative loading from Jcuint020 (-0.9661) as they did not play any role in explaining other principal components.

Table 11: Eigenvectors, Eigenvalues and proportions of variability for four principal components among 18 SSR for 30 *Jatropha curcas* accessions

SSR primer	PC1	PC2	PC3	PC4
Jatr002	0.0000	**0.9540**	0.0000	0.0000
Jcuint020	0.0000	0.0000	0.0000	**-0.9661**
Jatr691	**-0.9841**	0.0000	0.0000	0.0000
Jcuint180	0.0000	**0.9722**	0.0000	0.0000
Jcuint143	0.0000	0.0000	**0.9402**	0.0000
Jcuint018	0.0000	**0.9540**	0.0000	0.0000
Jatr159	0.0000	0.0000	0.0000	0.0000
Jatr115	**-0.9701**	0.0000	0.0000	0.0000
Jatr698	0.0000	**0.8967**	0.0000	0.0000
Jatr845	**-0.9701**	0.0000	0.0000	0.0000
Jatr739	0.0000	0.0000	0.0000	**0.8894**
Jcuint158	**-0.9701**	0.0000	0.0000	0.0000
Jatr209	**-0.9841**	0.0000	0.0000	0.0000
Jatr704	0.0000	0.0000	0.0000	**0.9169**
Jcuint115	0.0000	0.0000	0.0000	0.0000
Jcuint060	0.0000	0.0000	**0.9143**	0.0000
Jcuint048	0.0000	0.0000	**0.9143**	0.0000
Jatr684	0.0000	0.0000	**0.0000**	0.0000
Eigen value/latent root	4.760	3.56	2.55	2.42
Proportion	26.445	19.83	14.19	13.45
Cumulative percentage	26.445	46.27	60.47	73.93

Bolded face indicate relevant markers when explaining the component

4.3. Correlation between morphological and molecular markers in characterization of *Jatropha curcas* accessions within Botswana

The genetic variability of the *Jatropha curcas* germplasm was assessed using a combination of morphological and molecular markers. The two sets of markers showed negative, positive and no correlation relationships. A total of eight morphological characters and six molecular markers showed moderate correlation (Table 13). The markers that showed positive correlation were peduncle base color with molecular marker Jcuint018 (0.63), Jatr691 (0.56) and Jatr209 (0.56). The seed size (length and width) correlated positively with molecular marker Jatr684 (0.66) and (0.58). The number of leaf lobes as well correlated positively with molecular marker Jcuint002 (0.56).

The negative correlations were observed between molecular marker Jcuint002 with leaf width (-0.68), leaf length (-0.54) and petiole length (-0.55). The leaf length also showed a negative correlation with molecular marker Jatr739 (-0.51). The number of male flowers per cluster also correlated negatively with molecular marker Jatr684 (-0.53). The rest of the markers used either showed weak or no correlation at all.

Table 12: Correlation among relationship estimates based on SSRs and morphological traits within *Jatropha curcas* accessions using the mantel test.

	Morphological Characters							
SSR markers	PBC	SL	SW	NLL	LW	LL	PTL	NMFpc
Jcu018	0.63							
Jatr691	0.56							
Jatr209	0.56							
Jatr684		0.66	0.58					-0.53
Jcu002				0.56	-0.68	-0.54	-0.55	
Jatr739						-0.51		

Key= PBC = petiole base color, SL= seed length, SW= seed width, NLL= number leaf lobes, LW= Leaf width, LL= Leaf length, PTL= petiole length and NMFpc number of male flower per cluster

CHAPTER 5

DISCUSSIONS

5.1. Characterization of *Jatropha curcas* accessions using Morphological Characters

Jatropha curcas is a polymorph species with variations reported in tree architecture, plant height, number of fruits, seed dimensions (length, width, lateral diameter) and seed weight (Guan *et al.*, 2013). Morphological characters such as seed traits, leaf traits, fruit traits and inflorescence traits, have been used by a number of researchers to investigate morphological variation in *Jatropha curcas* germplasm in Asia, South America, Central America and Africa (Guan *et al.*, 2013; Sharma and Kumar, 2013; Saadaoui *et al.*, 2015). Those traits were all found to be of great importance to distinguish genetic variability, and have led to a better classification of *Jatropha curcas* genotypes in the above mentioned regions (Sunil *et al.*, 2011; Sunil *et al.*, 2013).

In the current study the variability was also noticed in the morphological characters that were used to characterize the accessions as per discussion.

5.1.1. Quantitative Characters

The quantitative characters display a continuous distribution of phenotypes. The variability is associated with the segregation of multiple minor genes or polygenes which have small individual effects and influenced markedly by the environment (Paran and Zamir, 2003). In this study the highest coefficient of variation was observed in the number of female flower per cluster and number of male flower per cluster in almost all the accessions. This indicates a wider variation existing among the accessions for those characters. In addition low coefficients of

variation were revealed by number of leaf lobes, seed length, and number of seed per fruit and this indicates a narrow variation exists among accessions for those characters. Such wide variation in the species was not surprising because those characters are predominantly influenced by environment (Ovando-medina *et al.*, 2011). The trend has been reported previously by several authors for different characters in different population of *Jatropha* (Kaushik *et al.*, 2007; Guan *et al.*, 2013 and Saadaoui *et al.*, 2015).

The analysis of variance for quantitative characters also revealed high significant differences ($P<0.05$) among the thirty accessions for peduncle length, number of seed per fruit, length of fruit stalk, fruit length, fruit width, seed length and width, seed weight, leaf length and width, petiole length, total flower per cluster, female and male flower per cluster and number of fruit per, with the exception number of leaf lobes (Table 7). This indicates substantial amount of genetic variability among the characters for this set of accessions. This is in agreement with other researchers for instance in Tunisia Saadaoui *et al.* (2015) investigated five characters of *Jatropha curcas* (foliar surface area, leaf length, leaf width, petiole length and number of nodes) and all of them revealed high morphological variability ($p<0.05$) among the eight accessions. Similarly, Guan *et al.* (2013) found that five seed traits (seed weight, seed length, seed width, lateral diameter, seed length and width ratio) were significantly different among eight populations of *Jatropha curcas* in China. Sharma and Kumar, (2013) also reported significant variations in number of seeds/fruit and seed weight was higher as compared to fruit width, seed width, fruit length and seed length in Himalayan *Jatropha curcas* germplasm.

5.1.2. Qualitative Characters

Qualitative characters have phenotypes that can be divided into discrete classes. They are controlled by single or a few major genes whose expression is not influenced markedly by the environment (Paran and Zamir, 2003). In this study qualitative characters revealed substantial amount of variability in petiole base pigmentation, flowering occurrence, female: male flower ratio, pigmentation on emerging leaves, and inflorescence compactness. Despite the substantial amount of variability revealed by those above mentioned characters invariant characters were also noticed among the accessions (fruit shape, flower color, and stem color) indicating that the characters failed to categorize the accessions. The pigmentation on emerging leaves was in line with what was observed by Silva *et al.* (2015) where the young leaves were distributed as red and purple.

5.1.3. Genetic Similarity revealed by Cluster analysis and Principal coordination analysis

Morphological cluster analysis was performed based on 15 quantitative and 5 qualitative characters and grouped the accessions into four clusters at dissimilarity coefficient of approximately 1.37. The Principal Coordination Analysis (PCoA) revealed exactly the same four groupings obtained by cluster Analysis. This indicated the existence of diversity among the 30 accessions for the morphological traits studied. The clustering pattern revealed by both two analysis techniques showed that some of the accessions were genetically distant as well as genetically close to each other. Despite the variability indicated by different clusters, a high level of relationship was observed among accessions with the same cluster. This closeness posed the possibility of duplicates among the accessions and suggests that they might have come from the

same genetic background. The distribution of accessions from the same collecting sites or regions into different clusters also indicated that the collecting sites were not related to genetic diversity. This was in accordance with what was reported by other authors in previous related studies. Rao *et al.* (2008) based on morphological traits and molecular marker studies respectively find out that, the tendency of accessions occurring in the same cluster cutting across the eco-geographical boundaries demonstrate that the geographical isolation need not necessarily be related to genetic diversity.

Similarly, Zapico *et al.* (2011) in their study, observed that no such association was detected for the *Jatropha curcas* accessions from various geographical origins. In another study by Subramanyam *et al.* (2009) the genetic diversity of wild and cultivated Indian *Jatropha curcas* provenances using RAPD markers, *Jatropha* accessions from different geographical regions shared 80% similarity. Furthermore, Gohil and Pandya, (2008) failed to establish a direct relationship between geographical distribution and genetic divergence using oil characters. However, it is not surprising for geographical origin of *Jatropha curcas* not to be associated with genetic diversity, according to Jain *et al.* (2003) there is possibility that, *Jatropha curcas* accessions from different geographical regions can be genetically similar. This genetic homogeneity can be attributed to similarities in agro-climatic conditions or seed movement (Subramanyam *et al.*, 2009).

5.1.4. Principal Component Analysis

Principal component analysis was performed based on 15 quantitative and 5 qualitative characters to further find out which traits were important in explaining the variation among the selected accessions. The first five principal components with eigenvalues of more than one were

selected, giving an accumulated total variation of 71.74%. The characters accounted for variation in *Jatropha curcas* were mostly the quantitative ones. In the first PC1, the variation was mainly due to leaf width, seed length, leaf length, fruit width, petiole length, fruit length, number of female flowers per cluster, seed width and number of fruit per cluster. In the second PC2, variation was mainly due to number of male flowers per cluster, total flower per cluster and female: male flower ratio. In the third PC3, variation was mainly due to length of fruit stalk and flowering occurrence. In the fourth PC4, female flower to male flower ratio caused the most variation. This proved that quantitative characters display a continuous distribution of phenotypes. Zapico *et al.* (2011) observed similar patterns in 13 *Jatropha curcas* accessions in the Philippines, where the leaf characters highly loaded in the first principal component and the seed characters loaded in the second principal component. Furthermore, Maurya *et al.* (2013) observed that the first principal component associated with seed length, seed width, seed weight/plant and number of seeds/plant. The previous and the current results indicate the need to take these characters into consideration when characterizing *Jatropha curcas* germplasm more especially when the resources are not permitting to consider all the characters.

5.2. Molecular characterization of *Jatropha curas* accessions using Simple Sequence Repeat (SSR) markers

Plant genetic variability is an important requirement for crop improvement through plant breeding. The first step in this process involves germplasm screening for establishment of genetic diversity (Maurya and Yadav, 2016). The use of molecular markers such as Simple Sequence Repeat (SSR) for study of genetic diversity in any food crop or tree crop requires selection and application of primers which will give clear, distinct, reliable and sufficient information required to study the variation that occur within the crop species (Arolu *et al.*, 2012). In this study Simple Sequence Repeat (SSR) markers were able to analyses the genetic diversity of *Jatropha curas* accessions growing in Botswana as per the discussion

5.2.1. Markers analysis

The polymorphic information content (PIC) provides an estimate of the discriminatory power of locus by taking into account, not only the number of alleles that are expressed, but also relative frequencies of those alleles (Boopathi *et al.*, 2008). PIC values range from 0 (monomorphic) and 1 very discriminative, with many alleles in equal frequencies (Boopathi *et al.*, 2008). In this study the PIC value of SSR markers in the present study was 0 for all the markers. This indicated that all the accessions were homozygous at these loci. This suggested that SSR markers used were not informative. The tendency of SSR markers amplifying only monomorphic bands in *Jatropha curcas* outside Mexico have been observed by several authors. Sun *et al.* (2008) studied the genetic diversity of 58 Chinese accessions using 16 microsatellite markers. The SSR markers amplified monomorphic bands which resulted in PIC value of 0. Montes *et al.* (2014b) observed

a PIC value of 0 for Asian and African *Jatropha curcas* germplasm which also confirm the current result.

Similar results were also reported by Vischi *et al.* (2013) where average PIC value was 0.3617 within the range 0.351 to 0.389. Furthermore, Yadav *et al.* (2011) reported that, fifty randomly selected EST-SSR markers were amplified in 25 accessions collected from different geographical regions of India. Twenty-one SSR markers were polymorphic and with allele variation from 2 to 4. The polymorphic information content value ranged between 0.04 and 0.61 indicating low to moderate level of informativeness of the SSR markers.

5.2.2. Genetic similarity or distances revealed by molecular cluster analysis and principal Coordination Analysis

Multivariate cluster analysis based on genetic similarity coefficient matrix was carried out using the UPGMA method in NTSYSpc version 2.1. Four clusters were resolved at a cut off of 0.85 genetic similarity coefficients. Principal Coordination Analysis (PCoA) revealed exactly the same four groupings obtained by cluster analysis. The clustering patterns showed that some of the accessions were genetically distant as well as genetically close to each other. The first cluster (A) is comprised of one accession of Ghana. It was clearly distinguished by the molecular markers showing that it is genetically distant from other accessions. The assumption was that the accession might have gone through breeding processes which might have altered its genetic makeup.

The second cluster (B) comprised of 27 accessions which are genetically close to each other. These accessions were collected from different villages with an extensive latitudinal range. This indicated that indeed the collecting sites were not related to genetic diversity. In this analysis, 18

of the SSR markers failed to distinguish 11 accessions from each other (BJC17, BJC79, BJC19, BJC20, BJC25, BJC27, BJC34, BJC45, BJC51, BJC57 and BJC62) suggesting a possibility of duplicates. This also suggested that the *Jatropha curcas* in Botswana has likely experienced a widespread dispersion by human intervention across the country either by seeds and vegetative materials. Such trend of no microsatellite variation in the *Jatropha curcas* genome is not limited to the current study as it was reported by authors. For instance Vischi *et al.* (2013) used 28 microsatellites; no microsatellite variation was detected among *Jatropha curcas* accessions collected from places outside Mexico. This strongly supported the fact that there were no microsatellite variations in the genome of *Jatropha curcas* outside its Centre of origin. Similarly, Yue *et al.* (2014) also assessed genetic variation using 29 microsatellites in 276 accessions of *Jatropha curcas* collected from 9 locations of South- America, Asia and Africa. Their results revealed that, all the 276 accessions were homozygous at all loci and shared the same genotype at each locus, also suggesting no microsatellite variation in the genome of *Jatropha curcas.* In Mexico, Basha et al. (2009) found that only accessions from Mexico and El Salvador showed variation by genotyping 12 microsatellite markers while the accessions from other countries could not be distinguished with those 12 SSR markers.

Despite the above, the third (C) and fourth (D) clusters comprised accession (BJC14 and BJC22) which were genetically distant from the rest. This clearly shows that the accessions were distinguished by the SSR markers. This raised the expectation of finding unique germplasm that will be used as parental line for improvement despite the high similarity coefficient index which ranged from 0.71 to 1.00. Similar results have been reported by other authors in different parts of the world on *Jatropha curcas* where genetic diversity study was assessed with an array of molecular markers. Shen *et al.* (2010) studied 38 populations of *Jatropha curcas* collected from

China using 9 AFLP primer combinations and a low genetic diversity was found among the accessions. The similarity coefficient index calculated ranged from 0.86 to 0.97 indicating a higher level of genetic similarity among the accessions. Similarly, Soonthornyatara *et al.* (2015) used AFLP markers to evaluate the genetic diversity of 28 diverse germplasm of *Jatropha curcas* collected from distinct geographical areas in India and very high similarity index of about 0.86 to 1.00 was revealed also indicating a narrow genetic diversity among the 28 Indian accessions. Montes *et al.* (2014b) assessed the genetic structure and diversity in *Jatropha* germplasm with 54 SSR and 120 SNP markers in a diverse, worldwide, germplasm panel of 70 accessions. Also observed was a narrow level of genetic diversity among the African, Asia and South America germplasm whereas, the genetic diversity in Central America and Mexico genotypes were very high.

The narrow or low genetic diversity in *Jatropha curcas* growing in Botswana is not surprisesing because *Jatropha curcas* in Botswana was mainly propagated through cuttings and according to Ellstrand and Roose, (1987) the plant species with predominantly vegetative reproduction generally, have lower level of genetic diversity than species that successfully produce solely by sexual reproduction. Another reason could be that the time it was introduced into Botswana was not long enough to give rise to genetic variation.

5.2.3. Multivariate Molecular Principal Component Analysis

The sign (positive or negative) tells the direction that a given variable in that PC is going on a single dimension vector. The negative sign indicates: inverse relationship between factor and variable but magnitude tells the strength of relationship. In this study Principal component analysis was performed based on 18 SSR markers to further find out which markers are important in explaining the variation among the selected accessions. The first four principal components with eigenvalues of more than one were selected, giving an accumulated total variation of 73.91%. The accumulated total variation were due to contributions from 15 SSR markers which either loaded negatively or positively in those first four principal components. These 15 SSR markers were associated with PC1, PC2, PC3 and PC4 did not play any role in explaining the variation on the entire principal components that were not selected. This was clearly indicated by having a value = 0 for those PCs. Also each of the selected principal components was associated with different SSR markers which did not play any role in explaining the variation beside the components it was associated with.

This result was also supported by Arolu *et al.* (2012) on molecular characterization of *Jatropha curcas* germplasm using inter simple sequence repeat (ISSR) markers in Peninsular Malaysia. In their study, only three principal components were also retained and accounted for 75.45% of the accumulated total variation. In addition to that, in a study that was carried out on 36 varieties of *Jatropha curcas*, 68.88% of total variation was observed with the varieties of two populations from Assam and Meghalaya in the Northeast India (Kumar *et al.*, 2011).

5.3. Correlation between morphological and molecular markers in characterization of *Jatropha curcas* accessions within Botswana

The finding of this study is that the correlation between the assays of microsatellites (SSR) and morphological markers is weak to moderate. The SSR markers used to assess molecular diversity indeed do not sample the same genomic regions with genes that control the expression of phenotypes. According to Laviola *et al.* (2012), this disparity could arise from the fact that neutral molecular markers, such as SSR, may be located in the non-coding regions of the genome. The other reason could be that, morphological traits are heavily affected by the environment when they are expressed, whereas molecular markers are not subject to such variation and their variation is based directly on DNA sequence variation (Collard *et al.*, 2005). Furthermore, according to Reed and Frankham (2001) correlation between the SSR and morphological markers could be reduced by the following factors; non additive genetic variation, different mutation rates, low statistical power and environmental effects on quantitative characters.

In previous studies weak relationships between genetic distances estimated from molecular data and genetic distances estimated from morphological data have been observed (Sunil *et al.*, 2011; Alves *et al.*, 2013). The accessions identified as the most diverse based on the analysis of eight phenotypic traits were not corroborated as diverse by molecular analysis which agrees with the current results (Sunil *et al.*, 2011). With the current results the outlier accessions in molecular analysis were also not unique based on morphological data. Similarly, Alves *et al.* (2013) assessed 117 accessions using a combination of phenotypic and molecular data (RAPD and SSR) and the correlation between the phenotypic dissimilarity matrix and the genotypic dissimilarity matrix was low.

The weak correlation between the molecular and morphological data matrices beside *Jatropha curcas* have been observed by a number of authors. For example, Hamza *et al.* (2012) did not detect any significant correlation between morphological data and molecular data (SSR markers) on genetic diversity of Date palm. Similarly Reed and Frankham (2001) also observed a weak correlation (r=0.217) between molecular and quantitative traits.

In a nutshell, the clustering based on morphological characters does not match that of groupings derived through molecular analysis. The dendrograms produced based on the two techniques differ. The morphological markers revealed slightly higher genetic distance estimates ranging from 0.51 to 2.48 while the molecular marker showed relatively smaller genetic distance estimates ranging from 0.71 to 1.00 and lower genetic diversity. The main reason of mismatch between clustering based on molecular markers and morphological markers may be that most of the characters are controlled by polygenes and are highly influenced by environment (Dalamu *et al.*, 2012). However, there is some striking similarities between the SSR markers and the morphological markers cluster analysis; there was largely a clear demarcation between the Ghana accession (GJC) and the Botswana accessions. The Ghana accession (GJC) is found to be an outlier in both morphological and molecular clusters analysis. These results suggest that the two approaches are generating different pattern of genetic diversity, and as such cannot be used as a surrogate for each other.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The principal component analysis reduced the original data from 20 into five principal components which accounted for 71.74% of the cumulative variance. This was mostly the contribution from the quantitative traits (leaf width, seed length, leaf length, fruit width, petiole length, fruit length, number of female flower per cluster, seed width, number of fruit per cluster, number of male flower per cluster, total flower per cluster, female: male flower ratio length of fruit stalk, and flowering occurrence). This suggests that quantitative traits should be taken into consideration when characterizing *Jatropha curcas* morphological.

Morphologically there was a substantial amount of variation found in the 30 studied *Jatropha curcas* accessions. This was revealed by higher coefficient of variation displayed by the accessions for those quantitative characters as well as highly significant differences ($P<0.001$) among accessions in 15 quantitative characters, except number of leaf lobes. Qualitative characters also revealed variability in petiole base pigmentation, flowering occurrence, female flower: male flower ratio, pigmentation on emerging leaves, and inflorescence compactness. Morphological cluster analysis was performed based on 15 quantitative and 5 qualitative characters and they grouped the accessions into four clusters at dissimilarity coefficient of approximately 1.37. This indicated the existence of diversity among the 30 accessions for the morphological markers studied. The principal coordination analysis revealed the same grouping pattern as that shown by cluster analysis and clearly separated accession GJC from the rest.

Molecular markers (18 SSR) revealed a narrow genetic diversity among 30 accessions of *jatropha curcas*. This was indicated by PIC values of zero for all the used markers and accessions with indistinguishable multi locus markers suggesting no microsatellite variation. Multivariate cluster analysis based on genetic similarity coefficient matrix revealed four clusters at a cut off of 0.85 genetic similarity coefficients and 90% of the accessions grouped in one cluster. This was also confirmed by principal coordination analysis where the same clusters revealed by cluster analysis clearly separated GJC from the rest. The principal component analysis reduced the original data into four principal components which accounted for 73.91% of the cumulative variance. The SSR markers contributed to variation in the selected *Jatropha curcas* accessions were identified.

The correlation between the morphological and molecular markers was both moderately positive and negative correlation was revealed by six molecular markers and seven morphological markers, respectively. The rest of the markers neither showed a weak or no correlation at all. This indicates that the molecular markers sample the genomic section of DNA which does not code for the phenotypic expression for the quantitative and qualitative characters.

In general a range of observations was made in the current analysis of genetic diversity of *Jatropha curcas*. An overall relatively high level of dissimilarity was observed among the accessions for most morphological characters analyzed. This indicates better possibilities for genetic improvement of the crop through selection and cross breeding. Also a high level of genetic similarity was revealed by molecular markers and this indicates the possibilities of duplicates among the accessions. Finally, results demonstrated that the two techniques were

generating different pattern of genetic diversity, and as such cannot be used as a surrogate for each other despite some few striking similarities that were observed.

6.2. Recommendation

It is recommended that the results from this study be used to identify potential useful genetic resources that are divergent from materials presently in use for use in the improvement of the current germplasm. More SSR Markers are needed to test the undistinguished accessions identified.

CHAPTER 7

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11