BOTSWANA UNIVERSITY OF AGRICULTURE AND NATURAL RESOURCES

A dissertation submitted in partial fulfilment of the requirements for the Master of Science Degree in Crop Science (Agronomy)

ASSESSMENT OF GENETIC DIVERSITY OF MUNGBEAN GENOTYPES IN BOTSWANA USING AGRO-MORPHOLOGICAL TRAITS AND MOLECULAR MARKERS.

BY

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DECLARATION

I solemnly declare that the work presented in this thesis is the result of my own findings and has not been submitted in any other institution for any academic award. The information derived from literature has been acknowledged in the text and a list of references provided.

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February 2022

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DEDICATION

This research work is dedicated to my late mom Atsayang Kgokong, and my lovely children, Rethabile and Letso. To God be the glory

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First and foremost, I would like to thank God almighty for giving me the opportunity and guidance to do this work. My deepest appreciation goes to my supervisor Professor Joseph Adjetey most importantly for all the support and encouragement he gave through this journey. His motivation, constructive advices, and critical contributions to this thesis deeply inspired me. I would like to express my sincere gratitude to my co supervisor Dr. Goitseone Malambane for his invaluable suggestions and for guiding and training me in molecular techniques. I am also grateful for the financial assistance I received from to the Department of Crops and Soil Sciences, Botswana University of Agriculture and Natural Resources (BUAN) to undertake molecular work at the laboratory.

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ABSTRACT

Mungbean (*Vigna radiata L, Wilczek*), is one of the leguminous crops that plays an important role in improving national food security and sustaining livelihoods of small-scale farmers in the developing world. Despite its economic importance, it has not been fully exploited in Botswana due to insufficient or lack of information on genetic background of the cultivated landraces. Therefore, the main objective of this study was to evaluate genetic diversity of eighteen mungbean genotypes in Botswana using agro-morphological characters and molecular markers. Two experiments were conducted in greenhouse and field conditions and laid in a randomized complete block design (RCBD) with three replications. A total of 34 quantitative and 11 qualitative traits were assessed. The qualitative morphological data revealed variation among traits including; growth habit, leaf colour, raceme position, pod curvature, pod colour, seed colour and testa texture. Analysis of variance (ANOVA) showed significant differences ($P \leq 0.05$) in yield and yield components, photosynthetic rates, chlorophyll content and root characteristics among the genotypes. The relationships among the tested traits of the genotypes were identified by Pearson's correlation and principal component analysis and the first four principal components accounted for 66.7% of the total variation. NTSYS-pc software using Unweighted pair group method with arithmetic mean (UPGMA) generated a dendrogram that distinctly grouped the 18 genotypes into four major clusters based on qualitative and quantitative morphological traits. For molecular analysis, 15 SSR makers were identified and used to assess genetic diversity among mungbean genotypes. Polymorphic information content (PIC) values ranged between 0.23 and 0.99 with highest value observed in CEDG305 and DMB-SSR 182 and the allele number per locus varied from 1 to 3 with maximum allele number produced by primer LR7323B and CEDG264. The

dendrogram constructed based on the SSR markers through UPGMA grouped the genotypes into seven clusters and the grouping of genotypes was not related to their geographic origin. Two genotypes (E132 and E14) were similar while the third (E092) had distinctively different characteristics. Clustering based on morphological traits and SSR markers showed differences in total number of clusters and positioning of genotypes within clusters. These markers proved to be effective in determining polymorphism and could be useful in assessment of genetic diversity of mungbean and further exploited in planning and execution of future mungbean breeding programmes in Botswana. Genotypes E084, E116 and VC1482E outperformed control genotype (Mmelegi) based on various agro-morphological traits and by virtue of their promising performance, they have a great potential and can be used as parents in future mungbean programmes to provide progenies with high genetic variability. We conclude that there is genetic variability in mungbean grown in Botswana which can be harnessed for improvement of the crop in Botswana.

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ACRONYMS AND ABBREVIATIONS

AVDRC	Asian Vegetable Research and Development
BME	β-mercaptoethanol
BNPGRC	Botswana National Plant Genetic Resource Centre
Bp	Base pairs
BUAN	Botswana University of Agriculture and Natural Resources
cm	Centimeter
DAR	Department of Agricultural Research
DNA	Deoxyribonucleic Acid
Dntp	Deoxyribose nucleotide triphosphate
E	Transpiration rate
EtBr	Ethidium bromide
g	Gram
G_S	Stomatal conductance
IBPGR	International Board for Plant Genetic Resources
ISSR	Inter simple sequence repeats
mg	Milligram
mm	Millimeter
NTSYS	Numerical Taxonomy and Multivariate analysis
PCR	Polymerase chain reaction

PIC	Polymorphic information content
Pn	Net photosynthesis
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomized complete block design
RFLP	Restriction Fragment Length Polymorphism
Rpm	Revolutions per minute
SSR	Simple Sequence Repeats
TAE	Tris-acetate-ETDA
µl	Microliter
UPGMA	Unweighted pair group method with arithmetic mean
UPOV	International Union for the Protection of new varieties of plants
UV	Ultraviolet light
VPD	Vapour Pressure Deficit

CHAPTER 1

1.0 GENERAL INTRODUCTION

Agriculture plays a fundamental role in the economies of most countries especially developing ones like Botswana. However, to ensure that the contribution of agriculture is maintained or improved, some interventions are required and these include growing high value crops, expanding access to global markets and lowering food prices through increased production (Sivaraj *et al*., 2013). Crop diversity is the key in realizing the above interventions. Crop diversity is not only important in agricultural production but it is used as a determinant of the total biodiversity in an ecosystem (Matson *et al*., 1997). It helps plant breeders to develop more productive varieties with improved quality traits required by farmers and consumers (Sivaraj *et al*., 2013). Crop diversity encompasses several aspects such as crop species diversity, varietal diversity within crop species and genetic diversity within crop species. It is essential to use a wide range of genetic material, especially of legumes in order to increase agricultural productivity (Abna *et al*., 2012). Some of the agronomic benefits of crop diversification include pest and disease management as well as soil fertility improvement, which are mostly influenced by crop combination. The best way to safeguard genetic variation is to exploit the existing genetic diversity of crops and establish crops for future research and breeding purposes (Shyamalee *et al*., 2016).

Reliance on two or more of the major crops has inherent agronomic, ecological, nutritional and financial risks and is perhaps unsustainable in the long run, particularly in view of climate change (Ebert, 2014), hence exploiting genetic diversity could be an alternative way of adapting to climate change. It has been reported that climate change is posing the greatest threat to agriculture and

food security in the 21st century especially in countries of sub Saharan Africa (SSA) (Ludi, 2009). Recent investigations suggest that climate change will pose a threat through biotic and abiotic factors in crop systems threatening crop sustainability and yield production (Lin, 2011). As temperature rises and weather patterns shift, there will be changes in nutrient cycling and soil moisture as well as shifts in pest occurrence and plant disease. This will greatly influence production and food security and with greater diversity in spatial and temporal distribution of crops in the cropping system, resilience to biotic and abiotic stresses will be enhanced (Lin, 2011).

It is essential to know the factors that limit crop productivity, and to search for possible solutions in accessing all existing genetic variation within and outside the crop species (Alghamdi *et al.*, 2017). Genetic diversity through morphological traits can be used to assess phenotypic variation in growing environments, and are also used as tools for indirect analysis of genetic variability and diversity (Kaur *et al.*, 2016). Molecular characterization is also useful in understanding the evolutionary development and diversification within plant species and to reveal the genetic diversity within a given taxonomic group (Nath *et al.*, 2017). The genetic variation among genotypes of various legume crops is being eroded as traditional cultivars are being replaced by modern cultivars across the globe. For instance, there is destruction of the wild relatives, which threatens the breeding process for future generations (Shyamalee *et al.*, 2016). Thus characterization is an important step in germplasm classification as it enables plant breeders to exploit genetic variability and make useful combinations to develop new varieties (Degefa, 2016).

Mungbean (*Vigna radiata L, Wilczek*), also known as green gram or locally as "letlhodi" belongs to the family Fabaceae, subgenus *Ceratrotopis* the genus Vigna (Varma *et al.*, 2018). It is cultivated throughout Southern and Eastern Asia, Central Africa and some parts of China, South

and North America and Australia. The short duration, photo-insensitivity, and dense canopy make mungbean assume a special significance in crop diversification, intensification and conservation of natural resources and sustainability of production systems (Kaur *et al*., 2016). Mungbean plays an important role in nutrition in developing countries (Allahmoradi *et al*., 2011) and it is also considered to be rich in nutrients like vitamins, manganese, calcium and iron (Brishti *et al*., 2017). The crop has the ability to fix atmospheric nitrogen, thus plays a key role in maintaining soil fertility and ensuring sustainability of production systems particularly, in low input, and small scale agriculture (Varma *et al*., 2018). Dikshit *et al*. (2010) reported that the productivity of mungbean is around 350 kg per hectare and this can be attributed to narrow genetic base resulting in low yield potential and susceptibility to biotic and abiotic stresses. Mungbean yields are generally dependent on weather conditions, soil, cultural practices and variety (Unal *et al*., 2008).

The importance of this crop has attracted many researchers in East and Southeast Asia, Southern Asia, East and Southern Africa as well as West and Central Africa, and collaborating under the Asian Vegetable Research and Development Centre (AVRDC), the group that has been actively working on mungbean for several decades (Karthikeyan *et al*., 2014). About 7000 accessions of mungbean are being kept at AVRDC in Taiwan from which useful and valuable traits can be derived and improved through breeding programmes, to maximize their potential and improve livelihoods of resource poor farmers (Pataczek *et al.*, 2018).

1.1 Statement of the problem

Based on the information mentioned on the background of this study, mungbeans are highly nutritious as they are a source of affordable proteins and minerals with the potential to improve national food security by sustaining livelihoods of smallholder farmers. Despite its potential in the dry climatic conditions of Botswana, it has not been fully exploited due to insufficient knowledge about its genetic diversity in this country. Moreover, there is inadequate documented studies on the characterization and agronomic performance of mungbean landraces grown by subsistence farmers in Botswana. The knowledge gap presents a problem to the scientific community and plant breeders in tapping into the potential of this crop for improved productivity in Botswana. Furthermore, without proper characterization of the genetic diversity of this crop and maintaining these distinct landraces, they face a serious threat of genetic erosion in the long run with a possible loss of some desirable mungbean genotypes in the country.

1.2 Justification of the study

Despite having a good potential for sustainable agriculture, mungbean seems to be neglected and underutilized in the research programmes of this country. It has been cultivated by small scale farmers as a subsistence crop for ages, only to a limited extent, and little work has been done, resulting in inadequate documented information on this crop. The available mungbean landraces assembled by the Botswana National Plant Genetic Resource Centre (BNPGRC) have been collected from different locations in the country but have never been investigated in terms of their genetic diversity and hence the diversity of the materials held is unknown. A proper classification

of the available accessions would be a useful starting point to taking advantage of the beneficial traits in mungbean accessions for the improvement of the crop in Botswana.

Mungbean grows fast and matures quickly, and these traits make it more suitable to the semi-arid regions of Botswana where moisture is limiting for a long growing season. In most studies elsewhere, it has been reported as a relatively drought tolerant crop and it could possibly be grown, additional to commonly used crop species like maize and sorghum or intercropped with these crops for food. Furthermore, it possesses attributes that make it to produce reasonable yields with low agricultural inputs and marginal lands. Like any other leguminous crops, mungbean has the ability to fix atmospheric nitrogen when in symbiosis with rhizobium bacteria, thus enhancing fertility by increasing the organic matter content of the soil. With this ability, it also has the potential to mitigate the effects of climate change by reducing greenhouse gas emissions through the reduction in use of synthetic nitrogen fertilizers (Jensen *et al*., 2012). Furthermore, relevant research and breeding are required to convert native existing landraces into competitive varieties with wide adaptation and promising industrial potential.

1.3 Objectives

1.3.1 General objective

The main objective of this study is to examine the genetic diversity of mungbean accessions in Botswana held at the Botswana National Plant Genetic Resource Centre (BNPGRC), using morphological and agronomic characters, and molecular markers.

1.3.2 Specific Objectives

- Establish the genetic variability within local mungbean germplasm.

- To characterize mungbean genotypes using the agro-morphological traits and molecular markers and determine the relatedness of these traits.
- Evaluate agronomic performance of mungbean genotypes.

1.4 Research questions

- Is there any genetic variation among local mungbean genotypes?
- Are there any relationships among the characters of the studied genotypes?
- Most importantly, which characters have a positive relation with yield?

1.5 Hypothesis

Null hypothesis (Ho): There is no genetic variation within the mungbean germplasm studied.

Alternative Hypothesis (Ha): There is genetic variation within mungbean germplasm studied.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Origin, domestication and distribution of mungbean

Mungbean is known to have originated in India or the Indo-Burmese region where it has been cultivated for centuries as indicated by fossilized remains discovered in India dated 1500-1000 BC (Shanmugasundaram, 1991). Brink and Belay (2006) suggested that the primary gene centre of diversity of mungbean was the central Asian region with India as the probable centre of domestication. The archaeological evidence shows that mungbeans were first domesticated in south eastern India and western Himalayan foothills, where they grew as wild plants about 4,500 years ago (Fuller and Harvey, 2006). Paroda and Thomas (1988) reported that India has a rich diversity of wild *Vigna* which comprises 14 species. *Vigna radiata* belongs to the subgenus *Ceratotropis*, a relatively homogeneous and morphologically and taxonomically distinct group, primarily of Asian distribution (Swaminathan *et al*., 2012). The subgenus *Ceratotropis* consists of cultivated and wild relatives, *Vigna radiata var. radiata* and *Vigna radiata var. sublobata (Roxb),* respectively. Its presumed progenitor is the wild form (*Vigna radiata var. sublobata),* which is widely distributed across the tropics, indicating that west Africa, northern Australia and Papua New Guinea are the centres for its diversity (Tomooka *et al*., 2014; Degefa, 2016). Both the cultivated and wild forms of this crop also possess a very large pool of genetic diversity which is conserved in gene banks across the globe (Tomooka *et al*., 2014). The major genetic improvement centre of mungbean (AVRDC) which has the largest collection of mungbean germplasm in the world, maintains about 5,900 accessions (Somta *et al*., 2009; Shanmugasundaram *et al*., 2009) and

has released more than 110 mungbean cultivars in Asia and around the world (Ali and Gupta, 2012). Its cultivation has spread to many parts of the world ranging from the Asian subcontinent, some parts of Africa, USA and has been introduced in Australia recently.

2.3 Taxonomy

Mungbean also known as green gram or locally as "letlhodi" belongs to the family Fabaceae, subgenus *Ceratrotopis* the genus *Vigna.* It is a self-pollinating diploid grain legume (2n=22) and has a small genome size estimated to be 500 Mb (Varma *et al*., 2018). The Asian *Ceratrotopis* is the largest subgenus and comprises 23 species which includes the major domesticated crops viz; the mungbean (Vigna *radiata (L),* Wilczek*),* black gram (*Vigna mungo (L),* Hepper*),* rice bean *(Vigna umbellata* Thunb*),* adzuki bean (*Vigna angularis* (Wild), and moth bean (*Vigna aconitifolia* (Jacq). It has been documented that genus Vigna includes about 150 species; 22 species are native to India and 16 to Southeast Asia. The wild types of mungbean, which are usually smaller in all parts than cultivated types, are usually classified into two botanical varieties namely; *var sublobata (Robx)*Verdc, occurring in India, Sri Lanka, Southeast Asia, northern Australia and in tropical Africa from Ghana to East Africa, southern Africa and Madagascar; *var setulosa* (Dalzell) with larger orbicular stipules and dense long hairs on the stem, and occurring in India, China, Japan and Indonesia. The taxonomic status of the species is summarized in Table 2.1:

Table 2.1 Taxonomic classification of mungbean

Kingdom	Plant kingdom
Division	Spermatophyta
Sub-division	Angiospermae
Class	Dicotelydeoneae
Order	Leguminoseae
Family	Phaseoleae
Tribe	Papilionoideae
Genus	*Vigna*
Sub genus	*Ceratotropis*
Species	*Radiata*
Variety	*Radiata*

Source: Shanmugasundaram,1991

2.4 Botanical description

The mungbean is an annual, semi erect to erect or sometimes twining, deep rooted herb, 30-120 cm tall. The central stems are more or less erect while side branches are semi erect (Mbeyagala, *et al.*, 2017a). The root system consists of a well-developed taproot with deeply placed lateral roots and leaves are alternate and trifoliate, or sometimes with five leaflets. Leaflets are medium to dark green, broadly ovate, sometimes lobbed, rounded at the base and pointed at the apex, 5 to 12 cm long, and 2 to 10 cm wide. Both the stems and leaves are covered with short brownish hairs. The

flowers are greenish to bright yellow and are self-fertile and highly self-pollinated. Flowering is indeterminate and may continue over a period of several weeks if the plant stays healthy. Pods mature in about 20 days after flowering. The pods are linear, sometimes curved, round and slender with short pubescence and consist of 8-20 seeds grains per pod (Dahiya *et al*., 2015). The seeds are small and globe shaped, exhibiting a wide range of variation from yellow, yellow greenish, light green, shiny green, dark green, dull green, brown and purplish black marbled or mottled with black patches (Paroda and Thomas, 1987). The seed coat often has ridges, making the seed rough to touch (Brink and Belay, 2006). The white yellowish, flat hilum is not concave and germination is epigeal (Shanmugasundaram, 1991). The crop matures very early with special features including; high yield, good nutritive value, drought resistant features, the reasonable cost of production and the ability to stimulate striga without being parasitized (Degefa, 2016).

2.5 Soil and climatic requirements

Mungbean is a warm season crop that grows in a wide range of climatic conditions. According to Ahmad *et al*. (2015) this crop grows well with an average rainfall of 600-1000 mm, but it can survive with less precipitation and grows best at altitudes of 0-1600 m above sea level. However at elevations of more than 1,800 m above sea level, the mungbeans have very poor pod set and are prone to diseases, especially, powdery mildew (Karanja, 2016). Heavy rainfall reduces pod setting and development and increases vegetative growth (Tomooka, 1991). To ensure good yield, adequate rainfall is essential from flowering to late pod fill while waterlogging reduces the oxygen concentrations around the roots of submerged plants and restricts nodule activity and nitrogen fixation. Brink and Belay (2006) reported that mungbean withstands drought well by curtailing the period of flowering and maturation, but it is susceptible to waterlogging and sensitive to frost.

They are well adapted to a wide range of soils, including red laterite soils and sandy soils (Mbeyagala *et al*., 2017a) and are not tolerant to wet, poorly drained soils (Karanja, 2016). Fertile, sandy loam with good internal drainage are the best soils for mungbean cultivation (Umata, 2018). Mungbean does not tolerate saline soils and can show severe iron chlorosis symptoms and certain micronutrient deficiencies on more alkaline soils (Department of Agriculture Forestry and Fisheries, 2010). Heavy clay and saline soils restrict root growth. The soil should have a pH range of 5.8 to 6.5 since soils that are too acidic adversely affect the growth of the rhizobia and availability of some nutrients.

2.6 Biological nitrogen fixation

It is well known that nitrogen (N) is an essential nutrient for plant growth and productivity. Nitrogen in the soil is replenished and naturally made available to legumes through the process of nitrogen fixation, which converts atmospheric nitrogen (N_2) to ammonia (HN_3). As a legume, mungbean is endowed with the property of maintaining and soil fertility through N_2 fixation as well as conserving and improving physical properties of soil by virtue of their deep root system (Ali and Gupta, 2012) and this distinctive character is very important in developing countries where synthetic fertilizer cost are relatively high (Rubio and Ludden, 2008). A complex relationship between the host (legume) and rhizobia must exist for symbiosis to take place (Ahemad and Kibret, 2014). Mungbean can also be used as green manure or as a cover crop to improve soil fertility (Mbeyagala *et al*., 2017a). Das and Singh (2014) reported that mungbean can fix 31- 85 N ha^{-1} which will not only meet its N requirements but leaves enough N for the succeeding crop.

Rubio and Ludden (2008) stated that biological nitrogen fixation accounts for 67% of the total nitrogen fixed globally per annum and approximately 25% is derived from legumes. In the developing world, chemical N fertilizers are not commonly used by farmers due to their relatively high costs and biological nitrogen fixation is an alternative means that can supply nitrogen to the plants. Intercropping of a legume with a non-legume often significantly increases the amount of symbiotic N fixed by the legume and the total amount of N uptake by the joint components of the system e.g soybean / maize (Shivay and Singh, 2000), sorghum / mungbean (Rashid *et al*., 2004), maize / mungbean (Morgado and Willey, 2003). This complementary use of N resources in legume / non-legume mixtures often results in a yield advantage by increasing the overall productivity compared to growing sole crops. Results of intercropping studies by Latati *et al*. (2013) revealed that maize *(Zea mays L.*) intercropped with common bean (*Phaseolus vulgaris L.*) confirmed the advantage of intercropping legume/ non legume over sole cropping system as sustainable agriculture. Maize grain yield was significantly increased by intercrop effect and interspecific competition for nitrogen N_2 fixation also decreased.

2.7 World mungbean production

Mungbean is currently cultivated throughout South and Southeast Asia, including India, Pakistan, Bangladesh, Sri Lanka, Myanmar, Thailand, Philippines, Laos, Cambodia, Vietnam, Indonesia, Malaysia, South China and Taiwan. It is also grown to lesser extent in many parts of Africa, the United States of America, and has been recently been introduced in parts of Australia (Smýkal *et al*., 2015). Mungbean yields normally range from 350 to 2 250 kg ha^{-1} and are largely dependent on weather conditions, soil, cultural practices and variety (Yi-Shen *et al*., 2018).

According to Harouna *et al*., (2018) 90% of the world's mungbean production is in Asia, particularly in India where around 50% of the world's production is located. The total area under mungbean cultivation in the world is estimated at 6 million ha (Hou *et al*., 2019) and global annual production is 2.5 to 3 million metric tonnes. However, India alone accounts for 65 % of the world acreage and 54% of the world production (Sherasia *et al*., 2018) but consumes almost its entire production. The average yield is considered to be 379 kg ha^{-1} (Somta and Srinives, 2007 ; Pandiyan *et al*., 2011). The world's second mungbean producer is China with annual production of about 0.5 million ha and average yield of 2000 kg ha^{-1} followed by Myanmar (Nair *et al*., 2014). Other major mungbean producing countries in the region include; Thailand, Burma, Pakistan and Indonesia.

2.8 Uses and importance of mungbean

2.8.1 Nutritional value

Mungbean is one of the major edible legume and it has been widely cultivated in many Asian countries as well as Southern Europe and some parts of Canada and the United States (Yi-Shen *et al*., 2018). In comparison with other legumes like soybean (*Glycine max*) and kidney bean (*Phaseolous vulgaris L.*) munbean has a high nutrient value (Mubarak, 2005), (Table 2.2; Chemical composition of mungbean per 100 g). Like most legumes, mungbeans are relatively high in proteins and they are a good substitute for animal protein in many Asian diets as they contain high amounts of cheap protein (Keatinge *et al*., 2011; Sarkar and Kundagrami, 2016). The most important part is the seed as it is used in several food products, both as whole seed and in processed forms (Pataczek *et al*., 2018).

Table 2.2 Chemical composition of mungbean seeds per 100g

Water	9.1
Protein	23.9 g
Fats	1.2 g
Carbohydrates	62.6 g
Dietary fibre	16.3 g
Calcium	132 mg
Magnesium	189 mg
Phosphorus	367 mg
Iron	6.7 mg
Zinc	2.7 mg
Vitamin A	114 IU
Thiamine	0.62 mg
Riboflavin	0.23 g
Niacin	2.3 mg
Vitamin B6	0.38 mg
Folate	0.25 mcg
Ascorbic acid	4.8 mg

2.8.2 Animal feed

Mungbean can be used as a forage legume because it can produce a large amount of biomass and then recover after grazing to yield abundant seeds (Mohamed and El Kramany, 2005). Similarly, the raw or processed seeds and the straw or hay of the whole plant can be used as livestock feed (Pataczek *et al*., 2018). The forage yield ranges between 0.64 t ha^{-1} and 1.8 t ha^{-1} with protein content of 13 – 21% on dry matter basis while mungbean straw has a protein content of 9 – 12%

(Sherasia *et al*., 2018). Mungbean straw has a high protein content when compared to cereal straw (Zainelabdeen and Eltalib, 2014).

2.8.3 Medicinal value

Mungbean has been consumed for more than 2000 years in China as it is well known for its health benefits such as reducing gastrointestinal disorders and heat stroke, and clearing toxins from the body (Fayyaz *et al*., 2018). It is also considered to aid in reducing the cholesterol level in the body, manipulation of tumor cells and regulating blood pressure (Minh, 2014). Owing to its palatable taste and nutritional quality, mungbean has been used as an iron- rich whole food source for baby food and induces less flatulence hence well tolerated by children (Sandhu and Lim, 2008). Furthermore, starch in mungbean is highly digestible when compared to those in chickpea (*Cierarietinum*), pigeon pea (*Cajanus cajan*) and lentils (*Lens culinaris*) (Sandhu and Lim, 2008).

2.9 Genetic diversity

Genetic diversity has been widely used as a powerful tool in the classification of cultivars and the study of taxonomic status of various crop species for successful breeding programmes (Tamiru *et al*., 2007; Ahamed *et al*., 2014). Osawaru *et al*. (2015) defined genetic diversity as variation of inherited characteristics in a population arising from evolution, mutation, migration, domestication, plant breeding and selection. Genetic diversity plays a vital role in plant breeding through the development of new and high yielding varieties and protecting the productivity of such varieties by integrating genes for disease and insect pest resistance as well as tolerance to abiotic stresses (Tamiru *et al*., 2007). With respect to climate change, genetic diversity may also assist plant breeders in breeding climate resilient varieties with desirable traits like tolerance to new

insect-pest and diseases, extreme heat and cold and various air soil pollutants (Bhandari *et al*., 2017).

The study of genetic diversity in genetic resources is a critical factor for breeders to better understand the evolutionary and genetic relationships within crop species, and assist in selection of germplasm in an effective way to develop strategies to incorporate in their breeding programmes (Paterson *et al*., 1991; Lavanya *et al*., 2008). A successful breeding programme depends on the complete knowledge and understanding of genetic diversity within and among genetic resources of the available germplasm and enables breeders to choose parental sources that will generate diverse populations for selection (Esmail *et al*., 2008) particularly those at risk of extinction (Grassi *et al*., 2006). Assessment of genetic diversity of cultivated crop plants is very important to select proper genotypes (Patil *et al*., 2013) and can also provide valuable information in order to help plant breeders identify promising crosses in a commercial hybrid programme. There are different ways of estimating genetic diversity within species of plants which include morphological characterization techniques, molecular and biochemical techniques (Fongod *et al*., 2012; Osawaru *et al*., 2015).

Genetic variability, which is referred to as a heritable difference among cultivars is required within a population to facilitate and sustain an effective long term plant breeding programme. Hence, understanding the genetic variability present in a given crop species for the character under improvement is of importance for the success of any plant breeding programme and broadening the gene pool of crops (Ahmad *et al.*, 2011). The multivariate techniques, such as cluster analysis and principal component analysis may be efficient tools in the quantitative estimation of genetic variation (Garg *et al*., 2017). Introduction of exogenous materials which have a specific genetic

background from other regions may play a great role in widening the genetic base, and also, to maximize hybrid vigour (Ahmad *et al*., 2011). Lack of genetic diversity can potentially limit the ability of cropping systems to resist unknown pests or adverse environmental conditions (Fongod *et al*., 2012).

2.10 Genetic diversity analysis

Genetic diversity assessment can be done through a number of techniques, including the use of morphological, biochemical, and molecular markers. Historically, morphological markers were used to assess genetic variation but recently many molecular marker techniques have developed into powerful tools to assess genetic relationships among crop species (Datta *et al*., 2012), which range from morphological characterization to various DNA-based markers.

2.10.1 Morphological markers

Evaluation and utilization of germplasm are the key aspects in investigating the extent of available diversity effectively (Mohammadi and Prasanna, 2003) and this can be achieved through characterization. Germplasm characterization represents a crucial step for conservation and preservation of genetic diversity. Characterization is the exercise involved in the description of plant germplasm and allows quantification and structuring of the genetic variability in the germplasm (Bode *et al*., 2013). Moreover, the expression of highly heritable characters can be estimated through morphological markers (Osawaru *et al*., 2015). In order to utilize the mungbean gene pool for development of new varieties, an extensive characterization of the various germplasm collection that constitute the gene pools for the crop needs to be done to identify the useful genetic diversity (Mohammadi and Prasanna, 2003).

Plant breeders frequently use characterization data for the initial description and classification of germplasm, in order to select genotypes in breeding programmes (Krichen *et al*., 2012) and estimate genetic diversity and cultivar development (Tantasawat *et al*., 2010) because a breeding programme mainly depends upon the magnitude of genetic variability. Traditionally, morphological traits were among the first genetic markers used in germplasm characterization despite having a few limitations. They are commonly used to evaluate genetic variation and provide a simple way of quantifying genetic variation while assessing genotype performance under normal growing environments (Fufa *et al*., 2005). Furthermore, evaluation of the genetic variability in some morphological traits of economic interest could be useful in selecting suitable materials for crop improvement in breeding programmes (Dos Santos *et al*., 2009). The use of morphological markers is easy and cheap when compared to that of biochemical and molecular markers, for preliminary characterization and varietal identification. However, even though they are inexpensive, they are greatly influenced by environmental conditions and may not be completely reliable because the traits are limited in number (Fufa *et al*., 2005). Genetic diversity using morphological markers has been studied in mungbean (Datta *et al*., 2012; Waniale *et al*., 2014; Sarkar and Kundagrami, 2016).

Sarkar and Kundagrami (2016) evaluated twenty-three mungbean genotypes for eleven agro morphological traits using multivariate statistical analysis and a wide range of variation was exhibited. For all the traits evaluated, the highest variation was found in number of pods per plant and low variability observed for harvest index. Cluster analysis using unweighted pair group method with arithmetic mean (UPGMA) grouped the genotypes into five clusters which varied from two to nine genotypes per cluster.

Waniale *et al*. (2014) evaluated 35 mungbean accessions for several diverse traits for two cropping seasons at different locations in Uganda. Genotype by environment interaction (GEI) was significant ($P < 0.05$) for all the traits indicating inconsistent performance by some genotypes across two locations and two seasons. However, principal component analysis (PCA) and dendrogram revealed narrow diversity in the mungbean collection.

Twenty mungbean genotypes were evaluated based on morphological characters to determine genetic diversity through multivariate analysis (Abna *et al*., 2012). Morphological characters including plant height, number of fruiting branches per plant, number of pods per plant, number of pod clusters per plant, pod length, number of seeds per pod, 100 seed weight and total seed yield per plant were assessed. Analysis of variance showed significant differences among genotypes for all traits. The genotypes were grouped into three clusters using cluster analysis by unweighted pair group method with arithmetic mean (UPGMA) and the principal component analysis accounted for maximum variability when compared to Analysis of variance (ANOVA) and cluster analysis.

2.10.2 Molecular markers

Molecular makers have been proven to be suitable tools that can yield significant information that enhances the scope of using germplasm in the crop improvement programmes (Patil *et al*., 2013). Molecular marker techniques comprising of various DNA markers have been successfully used to monitor and characterize the genetic diversity of mungbean germplasm collections (Dhedhi *et a*l., 2015) and these include: Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and Simple Sequence Repeats (SSR). Unlike morphological markers, molecular markers are more

advantageous as they give precise results at a more detailed level without interferences from environmental factors. They are stable and present in all tissues regardless of stages of crop growth (Mondini *et al*., 2009). However, molecular markers are expensive to purchase thus limiting the size of samples used for analysis and their application might be laborious and expensive for very large collections of minor crop species. In this study, Simple Sequence Repeats (SSR) were used to study the genetic diversity of mungbean.

2.10.2.1 Microsatellites or Simple Sequence Repeats (SSR)

Simple sequence repeats (SSR) also known as microsatellites are repeats of short nucleotide sequences DNA motifs of 1–6 base pairs (bp) in length, and widely distributed throughout the eukaryotic and prokaryotic genomes (Parida *et al*., 2008). They are codominant markers of relatively small size, which can be easily amplified with the polymerase chain reaction (PCR) (Chistiakov *et al*., 2006).

The SSR markers have emerged as the most widely used molecular markers and are gaining popularity owing to their more desirable genetic attributes. They have been regarded as one of the most effective and valuable molecular markers among numerous molecular markers due their codominant inheritance, multi-allelic nature, relative abundance, high stability and high rate of polymorphism. In addition, they are highly reproducible, present anywhere in the genome both in coding and noncoding regions and require only small amounts of genomic DNA for analysis (Pérez-Jiménez *et a*l., 2013).

Microsatellite markers have been successfully applied in various aspects of molecular genetic studies including: DNA fingerprinting (Molla *et al*., 2016; Reflinur *et al*., 2016) assessment of

genetic diversity (Wang *et al*., 2018), genetic linkage mapping (Wu and Huang, 2007), marker-assisted selection (Kaur *et al*., 2018) and gene flow characterization (Perdereau *et al*., 2014). These markers are amenable to high-throughput genotyping thus suitable for paternity verification and construction of high density genome maps (Parida *et al*., 2008). They have also proven to be very effective for the authentication of food components, from both animal and plant origins (Scarano and Rao, 2014). With the aforementioned reasons, the SSRs are regarded as the markers of choice.

2.11 Molecular diversity studies on mungbean and other crop species

Various DNA markers have been used to determine genetic diversity and SSRs (Prasanna *et al*., 2019), RAPD (Datta *et al*., 2012) , ISSR (Singh *et al*., 2012) and AFLP (Bhat *et al*., 2005) have been used to study genetic diversity among mungbean germplasm. Prasanna *et al*. (2019) undertook a study to examine genetic divergence of the 19 randomly selected mungbean genotypes using 10 SSR primers. Analysis of variance revealed significant variations among all the traits studied thus indicating a wide range of variability among the genotypes.

Datta *et al*. (2012) studied 24 released Indian cultivars of mungbean to investigate and compare genetic diversity using random amplified polymorphic DNA (RAPD) markers. Percentage polymorphism ranged from 33 to a maximum of 100% and the results revealed narrow genetic base in cultivars used in the study. Singh *et al*. (2012) studied genetic diversity among 87 mungbean genotypes from India and neighbouring countries using ISSR primers. An average of 18.12 bands and 16.87 polymorphic bands per primer was obtained. A dendrogram grouped genotypes into 5 clusters and positive correlation was exhibited in both primer and number of polymorphic bands. Bhat *et al*. (2005) studied the extent of diversity among seventy-one cultivars

of mungbean using AFLP technique and the results revealed a narrow genetic diversity among the cultivars analysed. The narrow genetic base clearly indicated that there is a need to explore and exploit more numbers of germplasm for improvement of mungbean breeding programmes.

Several studies have also been reported on the genetic diversity of mungbean accessions at the international level through a combination of morphological characters and molecular markers (Handerson *et al*., 2014; Kaur *et al*., 2016) and for other crops. A combined use of both morphological and molecular methods of characterization can be appropriate to determine genetic diversity in order to obtain complementary results as revealed by different studies. Kaur (2016) studied genetic diversity of twenty-three genotypes of green gram through morphological characters as well molecular markers (RAPD, ISSR, and SSR). A total of 216 bands were produced, of which 190 exhibited polymorphisms. UPGMA dendrogram based on the combination of morphological and molecular markers divided the 23 genotypes into three main clusters. A similar study was undertaken by Handerson *et al*. (2014) in pea (*Pisum sativum L.)* genotypes using morphological and molecular markers and the researchers identified high levels of genetic variation among the genotypes. They also reported that diversity shown by molecular markers was higher compared to those of morpho-agronomic characters

In general, since many studies have revealed that the genetic information provided by morphological characters or assessment of genetic diversity based on morphological characters is not adequate, there is support for the use of morphological-molecular characterization simultaneously. This study focuses on the use of both molecular and morphological markers because each application provides complementary information that yields firm resolutions in genetic diversity analysis. Classification of the plant species still relies on morphological traits as

it is the initial step in germplasm evaluation making significant contributions to understanding genetic diversity. Molecular markers on the other hand have proven to be more convenient to reflect the genetic relationships in crop species. Therefore, molecular markers could be used with morphological characters to characterize the accessions more precisely since genetic variability within the evaluated characters commonly exists. This will also provide an opportunity for plant breeders to identify mungbean landraces with useful and desirable traits suitable for the climatic conditions of Botswana and further develop new and improved cultivars to be incorporated into crop improvement programmes, hence reducing continuous genetic erosion of the local mungbean germplasm.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Description of experimental site

The study was conducted at the Botswana University of Agriculture and Natural Resources (BUAN) and Department of Agricultural Research (DAR) in Sebele, Southern Botswana. The experimental site is located at latitude 24°39′16″ S, Longitude 25°54′30″ E, at an elevation of 994 m. The climate of this region is classified as semi-arid with hot relatively moist summers and cool dry winters. The area usually receives mean annual rainfall of 538 mm that is erratic and poorly distributed. Daily average minimum and maximum temperatures range between 16.5°C and 27.9°C during the growing season. The soils of the experimental site have been classified as shallow, sandy loam soils (Legwaila *et al*., 2012).

3.2 Plant material

A total of 18 mungbean accessions, representing the total number kept at the Botswana National Plant Genetic Resource Centre (BNPGRC), Department of Agricultural Research (DAR) was used in this study. One is a released variety from the Department and 16 accessions are landraces collected from farmers in different villages within the country, whilst the passport information of one is unknown. The released variety (Mmelegi) was used as the control (Table 3.1 and Figure 3.1).

Table 3.1 List of mungbean genotypes and their collection sites

Genotype	Village	District	Latitude	Longitude
E28	Mmathethe	Southern	25.28274	-25.31555
E20	Moshupa	Southern	25.43385	-24.78970
E17	Maboane	Kweneng	24.53778	-24.11878
E38	Gantsi	Gantsi	21.62442	-21.68896
E14	Mogobane	Southeast	25.28274	-21.68896
E084	Kgope	Kweneng	25.94157	-24.33033
E083	Pelotshetlha	Southern	25.33054	-25.20017
E092	Tatisiding	Northeast	27.47079	-21.28097
E130	Machaneng	Central	27.48288	-23.18493
E132	Mahalapye	Central	26.81889	-23.10636
E115	Palapye	Central	27.12022	-22.53819
E116	Bobonong	Central	28.42277	-21.97002
E122	Mokhomma	Southern	26.60125	-24.75645
E158	Tonota	Central	22.64474	-21.45310
E48	Hukuntsi	Kgalagadi	21.83295	-23.94047
TM0117	Kumakwane	Kweneng	25.86031	-24.66310
Mmelegi (released variety)				
VC1482E (unknown passport data)				

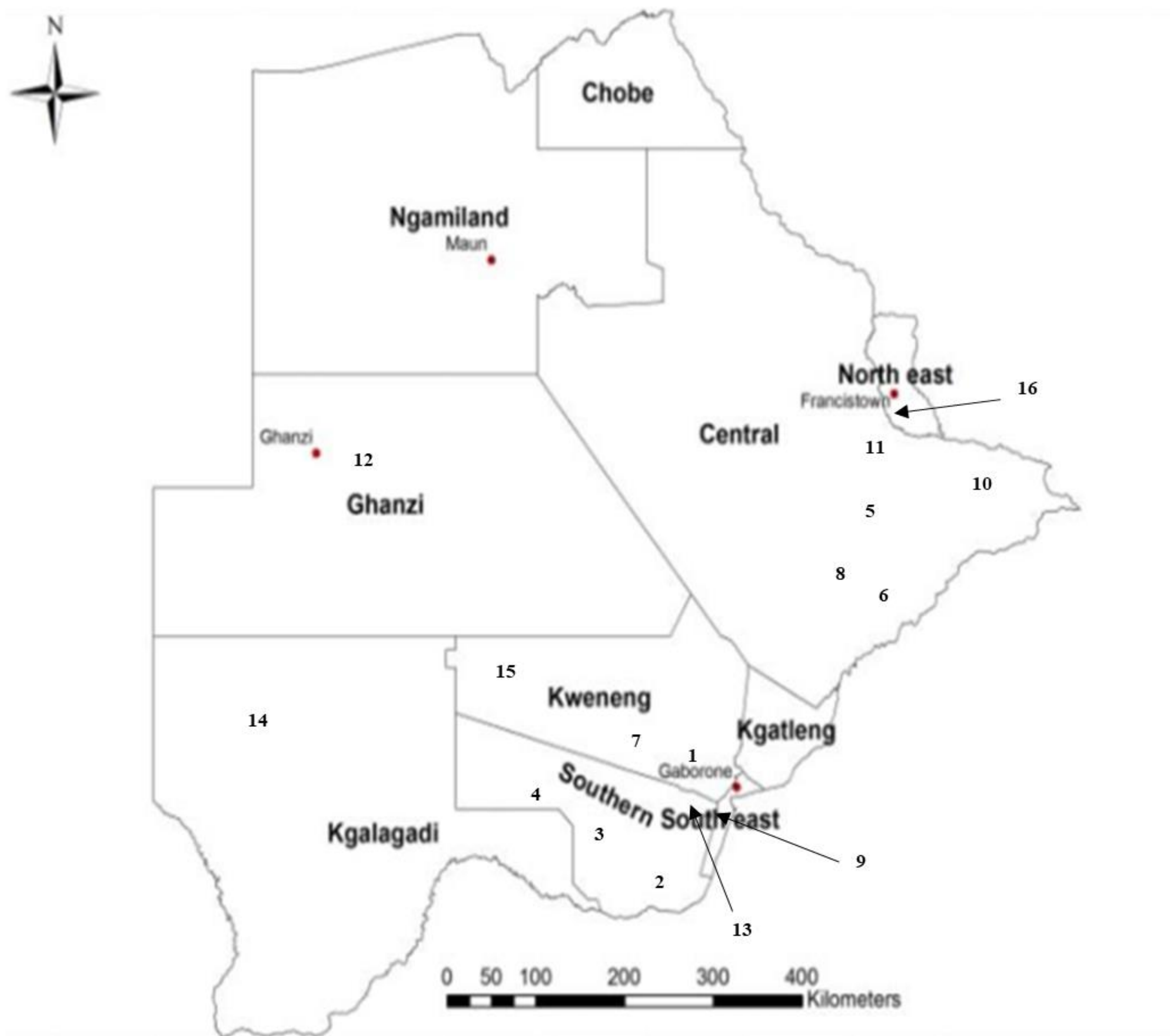

Figure 3.1 Map of Botswana showing the collection sites of mungbean genotypes

Key: 1; Kumakwane, 2; Mmathethe, 3; Pelotshetlha, 4; Mokhomma, 5; Palapye, 6; Machaneng, 7; Kgope, 8; Mahalapye, 9; Mogobane, 10; Bobonong, 11; Tonota, 12; Gantsi, 13; Moshupa, 14; Hukuntsi, 15; Maboane, 16; Tatisiding

3.3 Experimental set up and design

Two experiments were conducted in the two different growing conditions being greenhouse and field conditions. The experimental design used for both studies was a randomized complete block design (RCBD) with three replications.

3.3.1 Experiment 1 (field study)

A field experiment was conducted during the 2019/2020 growing season at the Department of Agricultural Research experimental field in Sebele to evaluate agronomic traits of the accessions under field conditions. Each experimental plot consisted of 2 rows of 5 m long, while the border plots had 3 rows each. The crop was grown under rainfed conditions, with no supplementary irrigation. The field plot layout and experimental field of the evaluated genotypes are illustrated in Appendices 1 and 3 respectively.

3.3.2 Experiment 2 (greenhouse)

A pot experiment was conducted in the greenhouse of the Department of Crop and Soil Sciences, Botswana University of Agriculture and Natural resources (BUAN), to study the morphology of the mungbean accessions and to collect plant samples for molecular characterisation. The same experimental design, was maintained in both experiments while observations measured differed per experiment. This experiment ran from August to November 2020. Growth and development of the evaluated genotypes in the greenhouse is presented in Appendix 2.

3.4 Soil preparation and planting

3.4.1 Experiment 1

Prior to planting, the land was prepared by ploughing, followed by harrowing to level the soil. This experiment was conducted under rainfed conditions without supplementary irrigation to mimic the natural conditions as much as possible. Two seeds were sown per hole at spacing of 75 cm x 20 cm (inter and intra row) giving a total of 25 plants per row.

3.4.2 Experiment 2

For the greenhouse experiment, soil was collected from the Department of Agricultural Research experimental farm in the same area where the field experiment was conducted. Five litre plastic pots, perforated at the base, were filled with soil and watered to field capacity before planting. Five seeds were sown at a depth of about 2.5 cm and later thinned to three seedlings per pot a week after emergence. The plants were watered to field capacity twice a week throughout the experiment.

3.5 Crop management

Throughout the growing season, all management practices were performed as per the general recommendations for mungbean (Department of Agriculture Forestry and Fisheries, 2010). Weeds were controlled manually by hand hoeing three times during the growth period. Plant protection measures were performed in the field experiment to safeguard the plants. During flowering and pod development stages, aphid infestation was observed and controlled by spraying with a mixture of Lebaycid 500 EC (fenthion) and RT buff (organic buffer system) using a knapsack sprayer. Ten

millilitre (10 ml) of Lebaycid 500 EC and 5 ml of RT buff were mixed together in 10 litres of water and sprayed on the plants.

3.6 Data collection

The morphological and agronomic characteristics were measured on quantitative and qualitative traits following descriptors for mungbean (IBPGR, 1980) together with guidelines described in the instructions of the International Union for the Protection of new Varieties of Plants (UPOV, 2009) selection of parameters and procedures for characterization. Two plants of each accession were tagged for data collection in the greenhouse experiment. Similarly, ten plants of each accession were randomly selected and tagged for data collection in each plot in the field. Qualitative traits were visually assessed and evaluated using different scoring scales while quantitative traits were measured using appropriate instruments.

3.6.1 Agro-morphological data collection

A total of 11 qualitative and 14 quantitative morphological traits, photosynthetic and shoot-root nodule traits were assessed. Qualitative data included; growth habit, growth pattern, leaf colour, terminal leaflet shape, flowering colour, raceme position, pod curvature, pod colour, seed shape a seed colour and seed texture. Some of quantitative traits included the following: days to flowering, days to 50% flowering, days to maturity, plant height, peduncle length, number of pods per peduncle, number of pods per plant, pod length, number of seeds per pod, 100 seed weight, grain yield per plant, grain yield per hectare and harvest index.

Table 3.2 List of qualitative characters and brief descriptions as listed from descriptor list (IBPGR, 1980).

Qualitative data	How data was collected	Character description and scoring
Growth Habit	Recorded on the 6th week of planting	2 Erect
		3 Semi erect
		4 Intermediate
Plant growth pattern	Recorded on the 6th week of planting	1 Determinate
		2 Indeterminate
Leaf colour	The colour of the leaves was visually observed and recorded	3 Light green
		5 Medium green
		7 Dark green
Terminal Leaflet shape	Recorded at 6 weeks of planting on young mature leaf	1 Deltoid
		2 Ovate
		3 Acute
		4 Cuneate
		5 Lobed
Flower colour	The colour was visually assessed	1 Light yellow
		2 Deep yellow
		3 Greenish yellow
		4 Others (olive green)
Raceme position	Recorded when peduncles reached full length	1 Mostly above canopy
		2 In the upper canopy
		3 Throughout the canopy
Pod curvature	Recorded from mature pods	0 Straight
		3 Least curved
		5 Medium curved
		7 Most curved
Pod colour	Recorded from mature pods	1 Straw
		2 Tan
		3 Brown
		4 Black
Seed shape	All observations were made on fully developed dry seed	1 Round
		2 Ovoid
		3 Drum shaped
		4 Other (specify)
Testa texture	All observations were made on fully developed dry seed	3 Smooth to rough
		5 Rough
		7 Rough to wrinkled
		9 Wrinkled

Table 3.3 List of quantitative characters and descriptions as listed from descriptor (IBPGR, 1980)

Quantitative Character	Description of character
Days to flower initiation	Recorded in days as number of days after sowing when plants in the plot set the first flower.
Days to 50% flowering	The days to 50% flowering was recorded after counting half of the plants per plot which has flowered.
Days to maturity	Recorded as number of days from sowing to when 50% of plants to have mature.
Plant height (cm)	Height was measured from the ground level to the tip of the raceme using a 1m ruler from the selected plants to determine the mean height of each plant.
Peduncle length (cm)	Recorded when peduncles have grown full length. Mean length of 10 peduncles, one from each of 10 randomly selected plants
Number of pods per peduncle	Recorded as mean of 10 randomly selected peduncles.
Number of pods per plant	Number of fully matured pods on each selected plant were counted and the mean value of the random sample plants was recorded as the number of pods per plant.
Pod length (cm)	Recorded as mean of 10 randomly selected pods.
Pod weight per plant	Recorded as average weight of the 10 randomly selected pods.
Number of seeds per pod	Recorded by counting the number of pods from the 10 randomly sampled pods and the mean recorded as number of seed per pod.
Hundred seed weight	100 seeds were randomly picked from each genotype per replication, counted and weighed and the average was then expressed as 100 seeds in grams.

3.6.2 Shoot, root and nodule traits

3.6.2.1 Number of nodules per plant

During the early flowering stage, any loose soil was washed away from the roots of the selected plants in the pots. Nodules were then detached from the root system and counted from each plant, then averaged to determine the number of nodules per plant.

3.6.2.2 Nodule fresh and dry weight

After nodules had been detached from the roots, they were weighed immediately to determine their fresh weight, then dried in the oven at 65ºC for 48 h to determine their dry weights. Before drying, ten nodules were chosen at random from the selected plants and each nodule was cut into two to determine their effectiveness by observing colour. Thus, the presence of a pink colour indicated effective nodules.

3.6.2.3 Shoot and root dry weight

After detaching the nodules from the roots, shoots were separated from the roots. Initial fresh weight of roots and shoots was measured, and samples dried in the oven at 65°C for 48 to determine their dry weights.

3.6.3 Grain yield per plant

To determine grain yield, plants were manually harvested at maturity from a 2.25 m^2 area in each plot (central two rows of each plot, 3 m long with a total of 30 plants), excluding the border rows. Grain moisture content was adjusted to 14% and the total weight of grains harvested from two

middle rows was averaged and expressed as grams per plant and then converted to kilograms per hectare (kg/ha).

3.6.4 Harvest Index

Harvest index was determined as the ratio of economic yield to total biological yield using the following formula by (Assefa *et a*l., 2013) ;

$$HI = GY / BGY * 100$$

Where;

HI - Harvest Index

BGY- Biological Grain Yield

3.6.5 Photosynthetic traits

3.6.5.1 Chlorophyll Content

A chlorophyll meter (SPAD-502, Konica- Minolta, Japan) was used to obtain readings estimating leaf chlorophyll concentration (SPAD value). One plant per pot in each replication was randomly selected and SPAD values were recorded from the fully matured leaves.

3.6.5.2 Photosynthetic and transpiration rate

To determine photosynthetic and transpiration rate, healthy and fully expanded leaves of the randomly selected plants within each plot, were measured using a handheld portable MultispeQ V

1.0 device used together with an android phone connected via bluetooth (Kuhlgert *et al*., 2016). The readings were taken from the onset of flowering, and this was done on sunny days between 09:00 and 12:00 am fortnightly.

3.6.5.3 Stomatal conductance

Stomata conductance was determined on the selected plants per pot. The measurements were performed between 09.00 and 12.00 am on sunny days using a portable leaf porometer (SC1, Decagon Devices, Inc., Pullman, USA).

3.6.6 Molecular Data collection

3.6.6.1 Sampling for DNA Isolation

Fresh leave samples were collected from the mungbean accessions grown in the greenhouse. The samples were snap frozen in liquid nitrogen and then stored in -80°C freezers until required for use. Total genomic DNA was extracted from each samples following the method developed by Dellaporta *et al*. (1983) with minor modifications as described below;

3.6.9.2 DNA Extraction following the Dellaporta Extraction protocol

Frozen samples were ground to fine powder in liquid nitrogen, placed in 2 ml tube containing 1.5 ml EB1(100 mM Tris pH 8.0, 500 mM NaCl, 50 mM EDTA pH 8.0, 700 µl β-mercaptoethanol (BME)). Four hundred (400) µl of 20 % SDS was added to each tube and about 1µl of RNase was added to degrade RNA. The samples were then incubated for 10 minutes at 65°C, vortexed and placed in ice for 20 minutes. Thereafter, the samples were centrifuged for 20 minutes at 15 000

rpm at 4°C. Seven hundred (700) µl of sample solution was transferred to a new Eppendorf tube, then DNA was precipitated from the supernatant by adding 500 µl of isopropanol, vortexed and centrifuged at 20, 000 rpm for 10 minutes, and the supernatant was discarded. DNA pellets were dried lightly and then washed twice in 500 µl of 80% ethanol and centrifuged at 20, 000 rpm for 5 minutes. Finally, the wash solution was discarded, and the DNA pellet obtained was dried overnight and re-dissolved in 100 µl elution buffer or DNA/RNA free H20.

3.6.6.3 DNA quantification

The DNA purity was assessed with a Nanodrop 2000 Spectrophotometer (Thermo Scientific) at wavelengths of 260 nm and 280 nm. Thereafter, the genomic DNA was confirmed by running the DNA samples alongside a 100 based-pair (bp) DNA ladder in 1% agarose gel. The gel was run for 1 h 30 minutes at 120 volts, soaked in a solution of ethidium bromide for 45 minutes and later exposed to UV light (trans illuminator) to visualize the bands and capture the image. The concentration of DNA was determined by comparing the intensity of bands with that of 100 bp molecular ladder.

3.6.6.4 Polymerase chain reaction (PCR) amplification

A total of 44 SSR primers were screened for polymorphism. The primer pairs were selected based on the polymorphisms as observed in the mungbean genetic diversity studies (Gwag *et al*., 2006; Sanghani *et al*., 2015; Kaur *et al*., 2016; Molla *et al*., 2016; Markam *et al*., 2018; Mathivathana *et al*., 2018; Kanavi *et al*., 2019). Thus, out of 44 primers screened, 15 gave scorable DNA fragments and later used to conduct the PCR. The names, nucleotide sequences and annealing temperatures of the selected primers are presented in Table 3.4.

Polymerase Chain Reaction (PCR) amplification for each primer set was performed in a 20µl reaction volume solution consisting of 11.5 µl DNA free water, 4 µl 5X KAPA Taq Extra Buffer, 2.4 µl $MgCl_2$, 0.4 µl dNTP, 0.5µl of each primer (forward and reverse), 0.05 µl KAPA Taq Extra DNA Polymerase and 1 ng template DNA. The PCR reactions were carried out as follows: initial denaturation at 95°C for 2 minutes, 35 cycles of denaturation at 95°C for 30 seconds, primer annealing for 30 seconds at respective primer annealing temperatures and 72°C primer extension for 1 minute. The final extension was then performed at 72°C for 5 minutes and lastly held at 4°C. The amplified PCR products were confirmed through 1.5 % agarose gel electrophoresis in 1X TAE (Tris-acetate-EDTA) buffer. The gels were run at 120 volts for 1 hour using 2 µl gel loading dye and then stained with ethidium bromide (EtBr). The bands were visualised under UV illumination and a photo taken using Gel Doc trans illuminator (Bio-Rad). The amplified products were weighed against 100 bp DNA ladder, which was used as the molecular marker for determining the product size of SSR primer.

Table 3.4 Primer sequence of the SSR primers used in the study

No	SSR primers	Sequence of primers (5'-3')
1	LR7322B	F: TCAGTCAGTGTCGATAGCATAGC R: GACACAGAGAGAGAGAGAGAGAAG
2	LR7323A	F: TGACGGAGAGAGAGAGAGAGAGAG R: TGCTTCCTTTTGTGTCTGAGTTAGAA
3	LR7323B	F: GCTATGCTATCGACACTGACTGA R: GCGCAAAGAGAGAGAGAGAGA
4	GBssr-MB77	F: GGAGAGGAACGAACAGGG R: GGCAGAGCATAACATGGC
5	VR 188	F: ATACAAGGGCAGGTGTAGCATC R: CAGAAAACTTCATCCCCAGCTA
6	VR 225	F: CAGCAACAGAACTTACAATCCCA R: CGGCAAATCCTCCTATATTCATT
7	Vr SSR 13	F: TTGATACGGCCACTTTCTCC R: CCATCAACGGTTTTTACGCT
8	Vr SSR 17	F: AACTTCGTCCTGCGCTTAAA R: AGCATGACCACACCAATCAA
9	CEDG006	F: AATTGCTCTCGAACCAGCTC R: GGTGTACAAGTGTGTGCAAG
10	CEDG092	F: TCTTTTGGTTGTAGCAGGATGAAAC R: TACAAGTGATATGCAACGGTTAGG
11	CEDG305	F: GCAGCTTCACATGCATAGTAC R: GAACTTAACTTGGGTTGTCTGC
12	CEDG264	F: GATTCCCTTCCTAGCTATGG R: CTGCTGGACATGAAGATTCAG
13	CEDG056	F: TTCCATCTATAGGGGAAGGGAG R: GCTATGATGGAAGAGGGCATGG
14	LR738A	F: CGCAAAGAGAGAGAGAGAG R: CCCCCATCTGAAAGAAAGAG
15	DMB SSR 182	F: TAGAGCCTTCTGGTTTTTCACA R: AGGAGGAGGATTTTGATGATGA

3.7 Data Analysis

3.7.1 Agro-morphological data analysis

Data collected on morphological quantitative traits were subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS) software version 9.1 program (SAS Institute Inc, 2004) and means separated using Least Significant Difference (LSD) at 5% level of significance**.** Descriptive statistical measures of mean and coefficient of variation were used to estimate variability within each quantitative trait. Pearson correlation analysis was also performed using the Statistical Analysis System (SAS) software version 9.1 program to assess the relationships between the studied traits of the mungbean genotypes at probability level of $P \leq 0.05$. Qualitative data were analysed using excel to generate frequency distribution tables to establish the percentage frequency of mungbean genotypes.

The multivariate techniques, the principal component and cluster analysis were done for the study of germplasm based on various traits (Mardia *et al*., 1995). Principal component analysis (PCA) was employed to analyse relationships among quantitative variables. The mean value of each trait for each genotype was subjected to PCA using the unweighted pair group method with arithmetic mean (UPGMA) and cluster analysis using the unweighted pair-group method with arithmetic mean UPGMA was also carried out based on the morphological traits. Each qualitative character was assigned a number i.e. 0,1,2,3 etc and coded as per observation.

3.7.2 Molecular data analysis

The amplified bands for simple sequence repeats (SSR) markers were scored as present (1) or absent (0) for each accession by manual inspection and this was used for the cluster analysis. Cluster analysis was performed on molecular data using unweighted pair-group method with arithmetic mean (UPGMA) to generate a dendrogram in NTSYS-pc software (Rohlf, 2000). Cluster analysis was used to investigate similarity and relatedness of genotypes, so that similar genotypes could be classified into one group and dissimilar ones into distinct groups (Somta *et al*., 2009). The markers were also assessed for the informativeness, by calculating polymorphism information content (PIC) for each marker using the equation by Roldán-Ruiz *et al*. (2000) ;

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

Where, pi is the frequency of the ith 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 number of alleles at each locus were calculated manually. PIC values give the information on the measure of usefulness of each marker in distinguishing one individual from another. The marker loci with a large number of alleles occurring at equal frequencies have the highest PIC values (Anderson *et al*., 1993).

CHAPTER 4

4.0 RESULTS

4.1 Qualitative morphological traits

The results of the two experiments (field and greenhouse) are presented together. In this present investigation, the following parameters; quantitative morphological traits, chlorophyll content, transpiration rate, net photosynthesis were measured from the field experiment while qualitative traits, shoot, root-nodule traits, and stomatal conductance were collected from the greenhouse experiment. Frequency distribution of qualitative morphological characters of mungbean germplasm is summarized in Table 4.1 and Table 4.2. Characters including growth pattern, terminal leaflet shape, seed shape and flower colour showed no variation while the remaining characters namely: growth habit, leaf colour, raceme position, pod curvature, pod colour, seed colour, and testa texture exhibited variations.

4.1.1 Vegetative growth parameters

Three classes of growth habit namely erect, semi erect and intermediate types existed among the mungbean genotypes (Table 4.1). The semi erect type was most dominant (61%) followed by erect types (28%) and only a few (11%) were intermediate (Plate 4.1). All (100%) the mungbean genotypes exhibited the indeterminate growth pattern and had deltoid terminal leaflet shape (Plate 4.2). Three different shades of leaf colours were observed, namely: light, medium and dark green. The medium green colour (72%) occurred more frequently compared to the light and dark green colours, with 17 and 11% respectively. Majority of the genotypes (about 67%) had their raceme in

the upper canopy and 33% had raceme positioned throughout the canopy. In this study only one flower colour (yellow) was observed in all the genotypes (Plate 4.2).

Table 4.1 Frequency distribution of the quantitative growth parameters of mungbean

Trait	Category	Frequency (%)
Growth Habit	Erect	28
	Semi erect	61
	Intermediate	11
Growth pattern	Determinate	100
Terminal leaflet shape	Deltoid	100
Leaf colour	Light green	17
	Medium green	72
	Dark green	11
Raceme position	In the upper canopy	67
	Throughout the canopy	33
Flower colour	Yellow	100

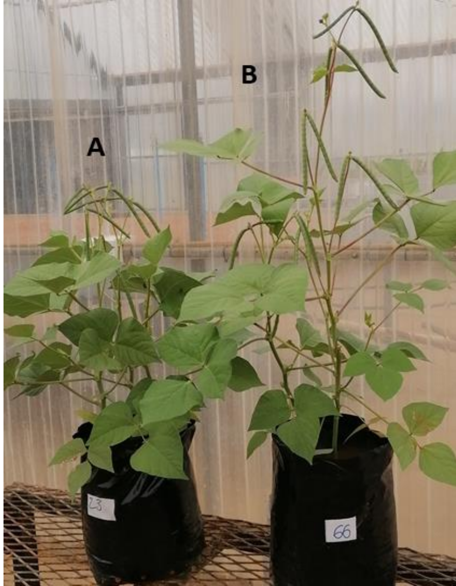

Plate 4.1: Variation in growth habit: (**a**) intermediate and (**b**) semi-erect

Plate 4.2: (**a**) Deltoid shape of mungbean terminal leaflet, **(b)** raceme positioned in the upper canopy and (**c**) flower colour (yellow)

4.1.2 Pod and seed traits

Some (61%) of genotypes had least curved pods while the others had straight pods (Plate 4.3) as described in the IBPGR (mungbean) descriptor (Table 4.2). Four different pod colours were observed namely: tan, brown, black and olive green (Plate 4.4). About 61% of the genotypes exhibited brown pod colour, 17% were olive green, and the black and tan colours were 11% each. There were two seed colours, with 94% being green and 6% being black (Plate 4.5). There was no variation in seed shape, and all the genotypes had drum shaped seeds. Testa texture was classified as rough, smooth to rough, and rough to wrinkled (Plate 4.5). Rough seeds were more frequent (66%) as compared to smooth to rough (28%) and rough to wrinkled (6 %).

Plate 4.3: Variation in pod curvature, (**a**) least curved and (**b**) straight pods

Table 4.2 Frequency distribution of the pod and seed related parameters of mungbean

Trait	Category	Frequency (%)
Pod curvature	Straight	39
	Least curved	61
Pod colour	Tan	11
	Brown	61
	Black	11
	Others (Olive green)	17
Seed colour	Green	94
	Black	6
Seed shape	Drum shaped	100
Testa texture	Smooth to rough	28
	Rough	66
	Rough to wrinkled	6

Plate 4.4: Variation in pod colour, (**a**) black, (**b**) brown, (**c**) tan and (**d**) Olive green

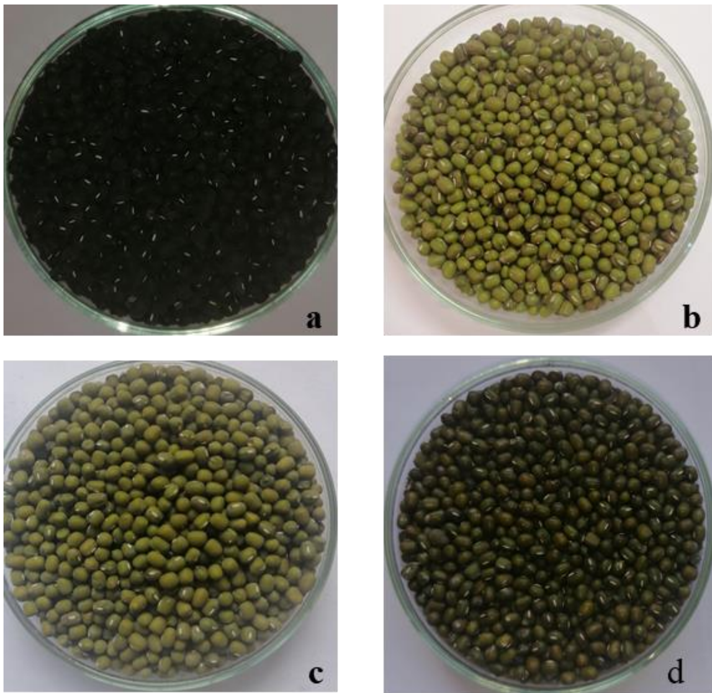

Plate 4.5: Variation in seed colour and testa texture, (**a**) black and **(b)** green, (**c**) rough and (**d**) smooth to rough

4.2 Quantitative morphological traits

4.2.1 Growth parameters

Comparisons for quantitative growth parameters namely: days to first flowering, days to 50% flowering, days to maturity, plant height, and peduncle length are presented in Figure 4.1 and Figure 4.2. There was a significant difference ($P \leq 0.05$) in days to flowering among the eighteen genotypes evaluated. The mean number of days to flowering ranged from about 41 days for the earliest genotypes (E084 and VC1482E, E084, E38 and E116) to about 84 days for the late ones (E28 and TM0117) (Figure 4.1). Significant differences were observed in the mean number of days to 50% flowering among the mungbean genotypes (Figure 4.2). Mean number of days to flowering ranged from 54 to 97 days. E132 had the highest (97 days) followed by E130 (96 days) while E084 had the least (54) number of days. The genotype, E116 was the first to reach maturity in 71 days while E 130 took 119 days to mature (Figure 4.3). Plant height ranged from about 50.97 cm for the tallest genotype (E115 and TM0117) to about 24.67 cm for the shorter ones e.g. (VC1482E and E116). The genotypes showed significant differences with respect to the mean peduncle length (Figure 4.4). E116 had the longest mean peduncle length of 10.13 cm while E28 had the shortest mean peduncle length of 3.3 cm.

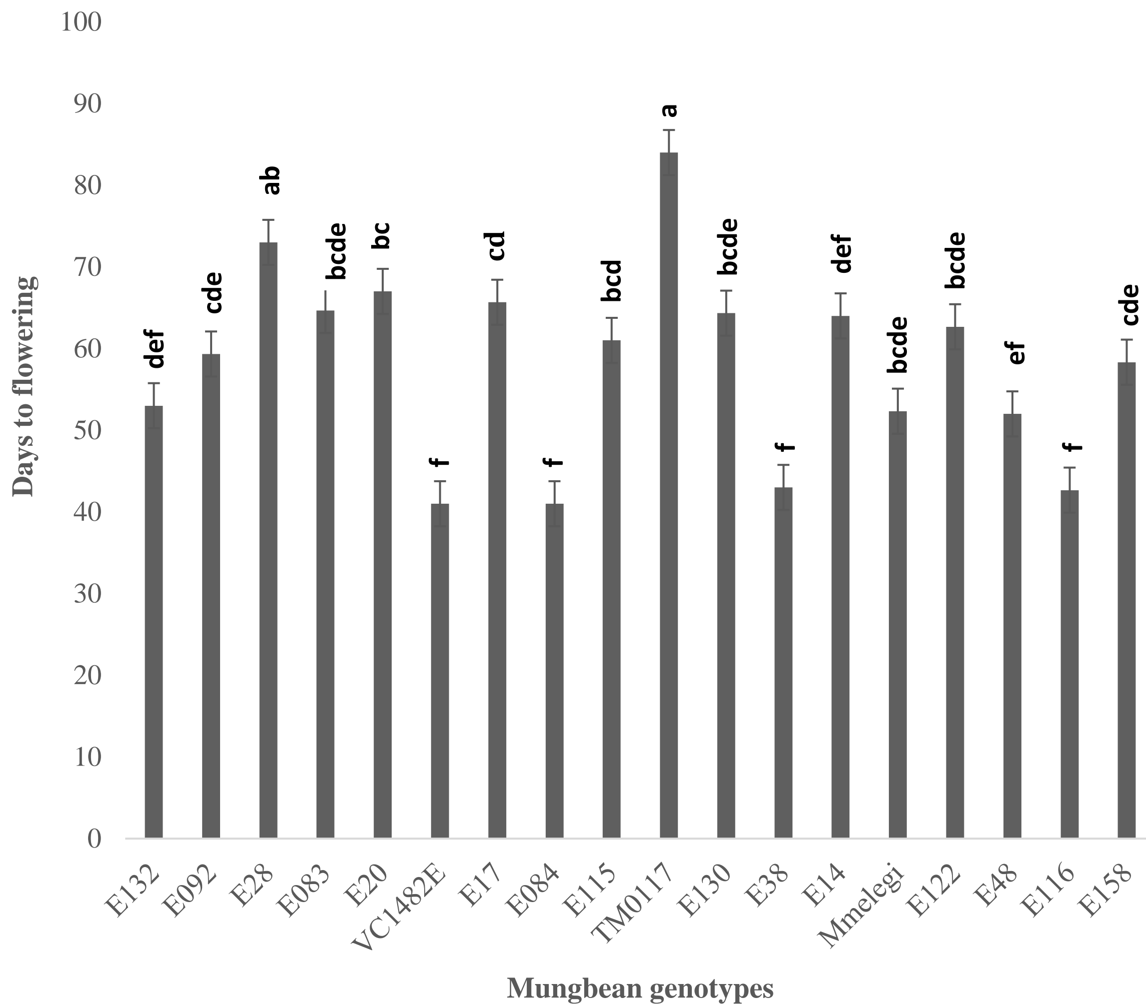

Figure 4.1 Mean comparison for days to flowering. Means are for N=3, with bars showing the standard deviation of the means. LSD test was performed at 5% significance level, and the significant differences among the values are indicated by the letters at the top of each bar

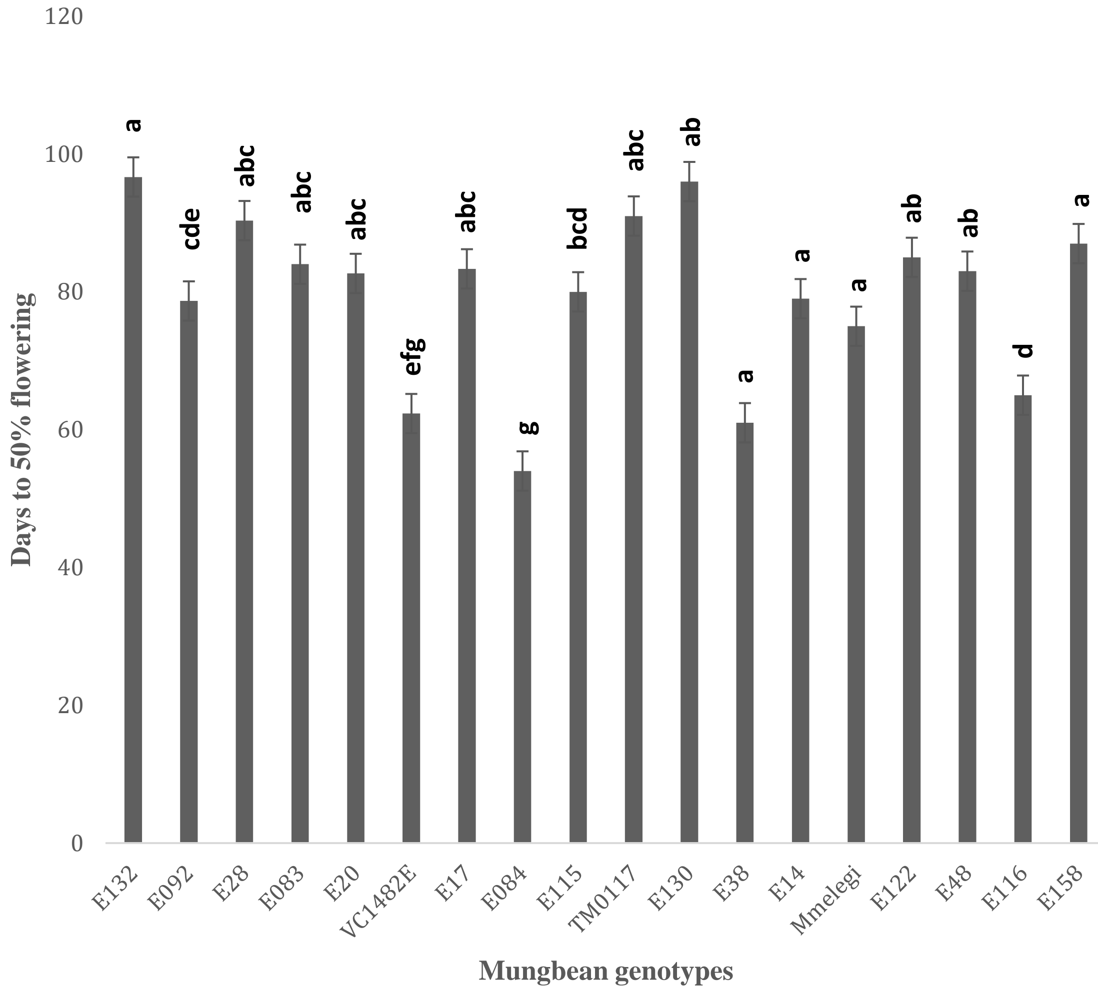

Figure 4.2 Mean comparison for days to 50% flowering. Means are for N=3, with bars showing the standard deviation of the means. LSD test was performed at 5% significance level, and the significant differences among the values are indicated by the letters at the top of each bar

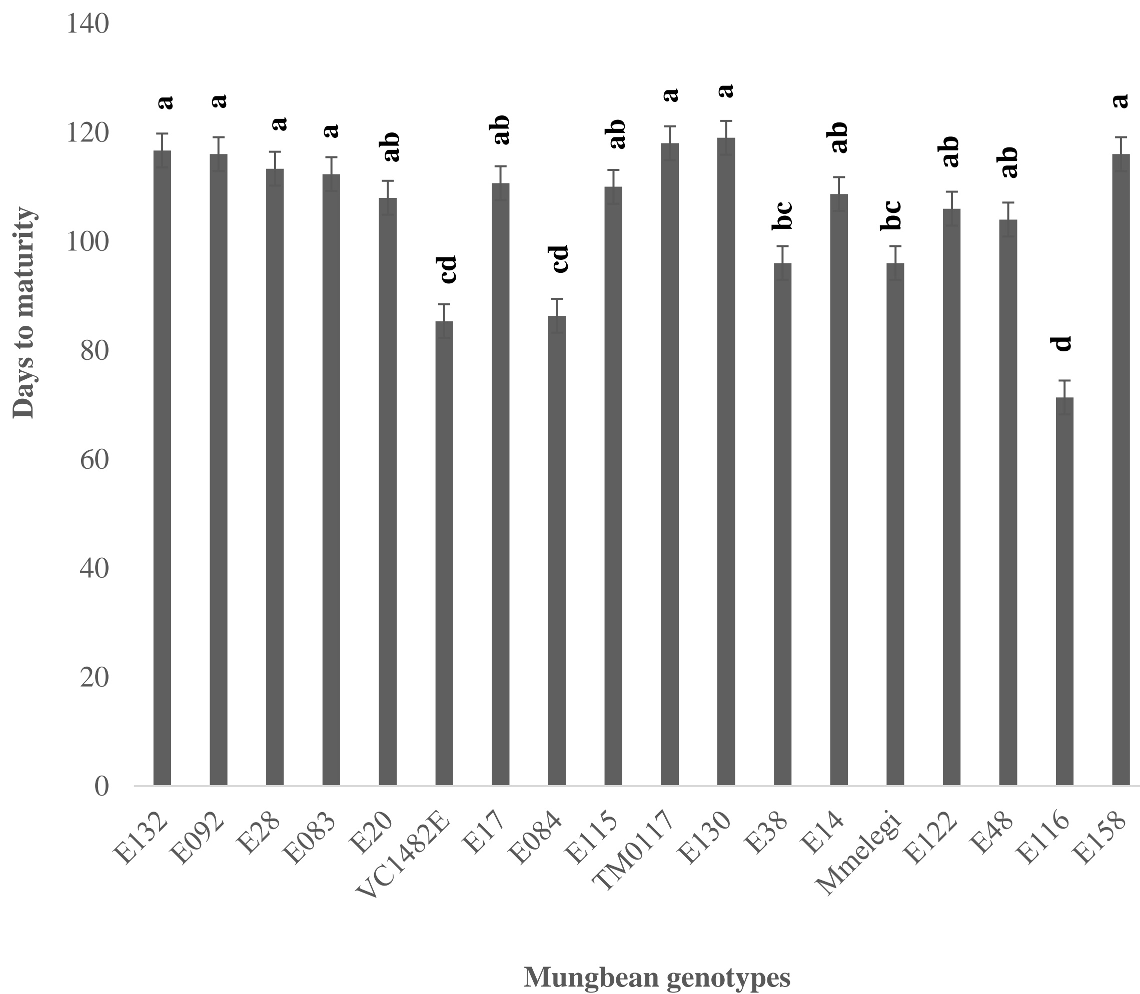

Figure 4.3 Mean comparison for days to maturity. Means are for N=3, with bars showing the standard deviation of the means. LSD test was performed at 5% significance level, and the significant differences among the values are indicated by the letters at the top of each bar

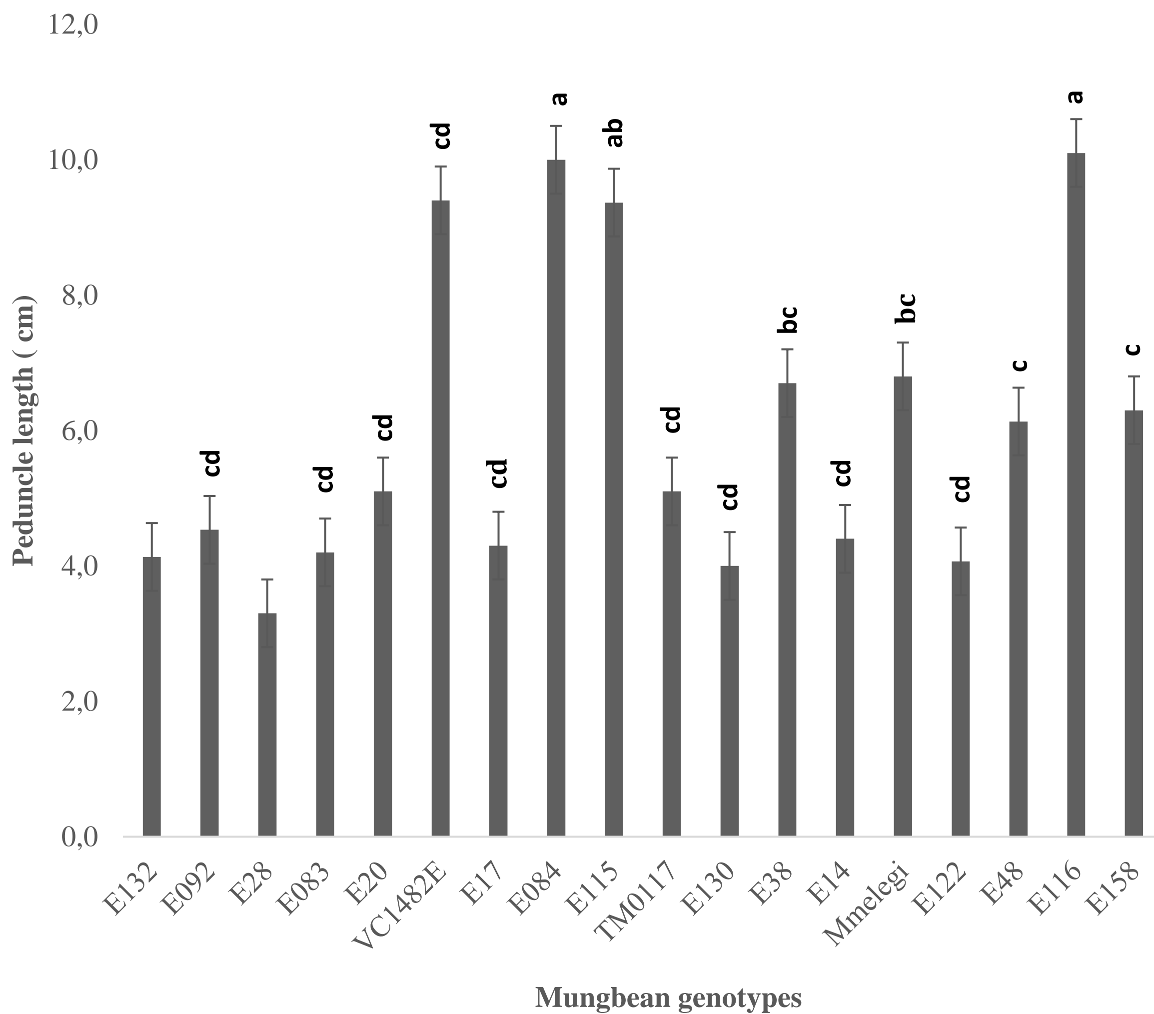

Figure 4.4 Mean comparison for peduncle length (cm). Means are for N=3, with bars showing the standard deviation of the means. LSD test was performed at 5% significance level, and the significant differences among the values are indicated by the letters at the top of each bar.

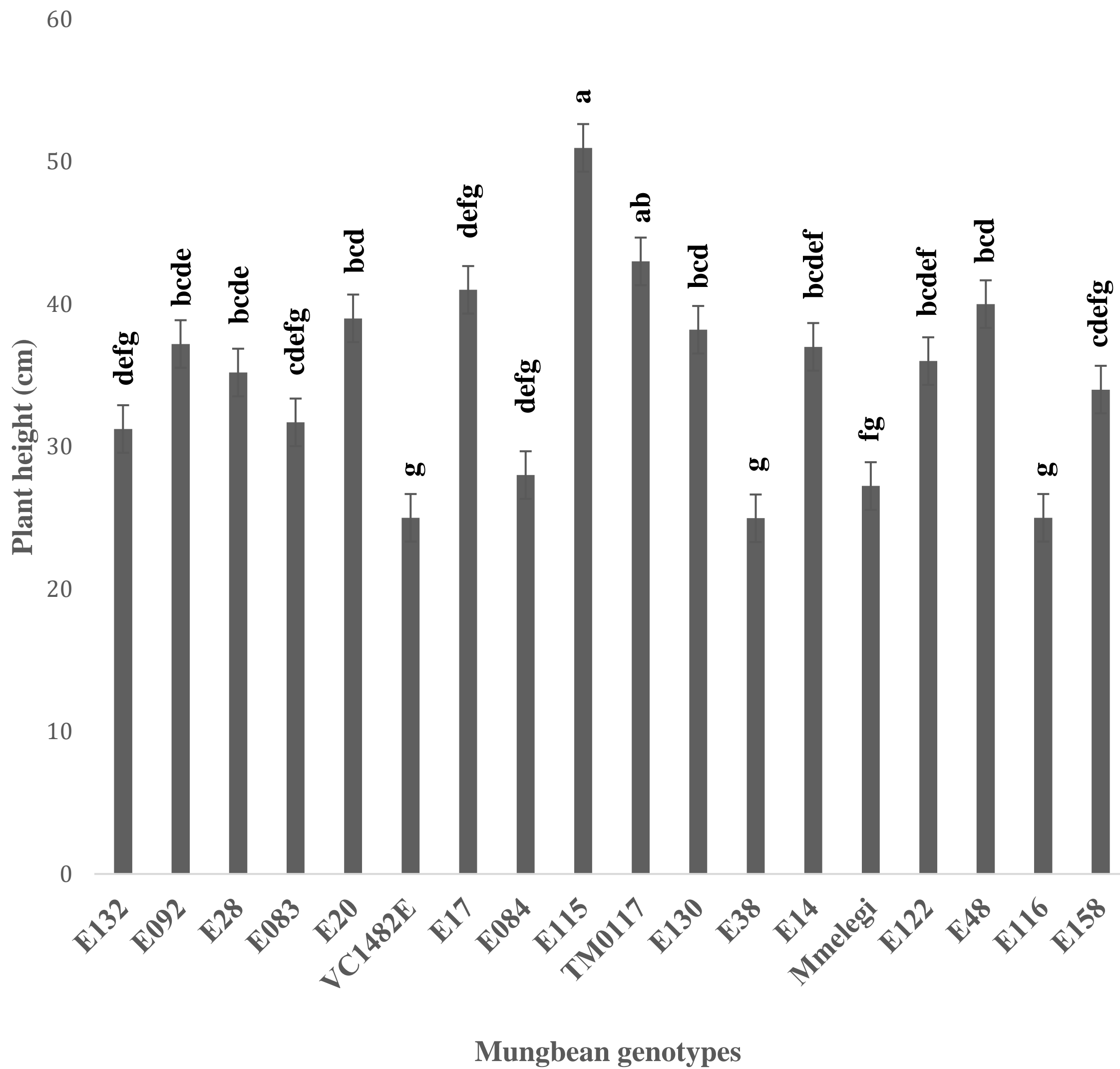

Figure 4.5 Mean comparison for plant height (cm). Means are for N=3, with bars showing the standard deviation of the means. LSD test was performed at 5% significance level, and the significant differences among the values are indicated by the letters at the top of each bar.

4.2.2 Yield and yield components

The results for yield and yield components of mungbean are provided in Table 4.3. Number of pods per peduncle was significantly different ($P \leq 0.05$) among the genotypes with the most prominent difference between genotype E20 (5) per peduncle, and E083 (3). Pod weight per plant varied significantly ($P \leq 0.05$) among genotypes with E130 recording the highest weight (13.5g) while the lowest was attained by E116 and E130 with 5.8 and 5.9g, respectively. Variation in number of pods per plant was significant ($P \leq 0.05$) and pod numbers ranged between 43 and 12. The highest number of pods per plant was produced by genotype E20, while genotype VC1482E, E130, E116 and E084 were statistically similar and had the lowest values. Three of these four genotypes (VC1482E, E116 and E084) were among the fastest to flower. Differences in pod length among the genotypes were significant ($P \leq 0.05$), varying from 9.2 to 5.7 cm. The longest pods were observed in genotypes E084 and E116, and the shortest in genotypes E130, E092, E28 and E122. The highest value for number of seeds per pod was recorded by genotype E122 with 11 seeds, while genotypes E130 and E115 had the lowest with 8 seeds. Grain yield ha^{-1} (Table 4.3) ranged from 71.9 to 290.8 kg. E116 had the highest grain yield while E115 recorded the lowest yield. Genotypes also displayed significant differences with respect to harvest index and values ranged from 7.6 % (E115) to 27.4 % (E116). Significant differences were also observed in 100 seed weight with genotype E132 recording the highest weight while E38 had the lowest. Grain yield per plant also varied significantly ($P \leq 0.05$) among the genotypes. The highest weight was recorded in E48 and the lowest in E158.

A wide range of minimum and maximum trait values together with considerable coefficient of variations were observed in most of the traits assessed (Table 4.4). Most variable traits had

coefficient variation (CV) values ≥ 41% and these included number of pods per plant with (CV = 50.1 %), grain yield per hectare (CV = 46.9 %), harvest index (CV = 45.7 %), peduncle length (CV = 45.4 %), grain yield per plant with CV = (41.2 %), while the CVs for the other reproductive traits were ≤ 25%

Table 4.3 Quantitative yield and yield contributing traits of mungbean genotypes

Genotype	NPP_d	NPP_t	PWP (g)	PL (cm)	NSP	100 SW (g)	GYP (g)	GY (kg/ha)	HI (%)
E20	5.3^{a}	43.0^{a}	13.5^{a}	6.8^{ab}	9.0^{abc}	5.3^{abcd}	5.8^{ab}	186.8^{abcde}	18.7^{bcde}
E28	4.7 ^{ab}	25.0^{bc}	11.^{ab}	5.8^{b}	10.3^{ab}	5.5^{ab}	6.3^{ab}	196.4^{abcde}	16.1^{cdefg}
E48	4.7 ^{ab}	24.0^{bc}	13.6^{a}	7.5^{ab}	9.7^{abc}	4.8^{abcd}	7.6^{a}	204.5^{abcd}	18.3^{bcde}
E092	4.3^{abc}	34.0^{bc}	6.1^{bc}	5.8^{b}	9.0^{abc}	5.2^{abcd}	3.6^{b}	129.6^{cde}	8.4^{gh}
E084	4.0^{bc}	13.0^{c}	7.2^{bc}	9.0^{a}	10.0^{abc}	5.8^{ab}	5.9^{ab}	185.8^{abcde}	18.9^{bcd}
TM0117	4.0^{abc}	22.0^{bc}	9.5^{abc}	6.2^{ab}	9.0^{abc}	5.4^{abcd}	5.0^{ab}	137.8^{cde}	9.2^{gh}
E14	4.0^{abc}	24.0^{bc}	9.7^{abc}	6.6^{ab}	9.0^{abc}	5.5^{abcd}	6.8^{ab}	163.4^{abcde}	13.3^{defg}
E122	3.7^{bc}	21.0^{bc}	8.2^{bc}	5.8^{b}	11.0^{ab}	5.4^{abcd}	6.3^{ab}	240.9^{abc}	20.0^{abcd}
E132	3.7^{bc}	19.0^{bc}	8.1^{bc}	6.4^{ab}	10.0^{abc}	6.2^{a}	4.5^{ab}	185.1^{abcde}	19.4^{bcd}
VC1482E	3.7^{bc}	12.0^{c}	7.2^{bc}	8.7^{ab}	9.0^{abc}	5.6 ^{ab}	4.7^{ab}	244.0^{abc}	23.6^{abc}
E130	3.7^{bc}	16.0^{c}	5.8^{c}	5.7^{b}	8.0^{c}	4.3^{d}	3.9^{b}	97.6^{de}	9.0^{gh}
Mmelegi	3.7^{bc}	18.0^{bc}	9.9^{abc}	7.8^{ab}	10.0^{abc}	5.1^{abcd}	6.4 ^{ab}	266.6^{ab}	24.2^{ab}
E116	3.7^{bc}	12.0^{bc}	5.9^{c}	9.2^{a}	9.0^{abc}	5.4^{abcd}	4.7^{ab}	290.8^{e}	27.4^{a}
E38	3.7^{bc}	19.0^{bc}	9.0^{abc}	7.6^{ab}	9.7^{abc}	5.6^{ab}	5.2 ^{ab}	186.5^{abcde}	15.4^{defgh}
E17	3.3^{bc}	27.0^{abc}	9.5^{abc}	6.8^{ab}	8.7^{abc}	5.5^{abcd}	5.4 ^{ab}	182.5^{abcde}	16.0^{cdef}
E115	3.3^{bc}	22.0^{bc}	6.6^{bc}	7.4^{ab}	8.0^{c}	5.0^{abcd}	3.9^{ab}	71.9^{e}	7.6^{h}
E158	3.3^{bc}	25.0^{bc}	7.5^{bc}	8.6^{ab}	8.3^{bc}	4.4^{cd}	4^{b}	141.7^{bcde}	11.0^{efgh}
E083	3.0^{c}	22.0^{bc}	9.^{abc}	6.7^{ab}	9.7^{abc}	5.4^{abcd}	6.2 ^{ab}	158.3^{bcde}	18.8^{fgh}

[a]Means followed by the same letter are not significantly different according to Least Significant Difference (LSD) at 5 % significance level. **NPP_d**: Number of pods peduncle^{-1}, **NPP_t**: Number of pods plant-1, **PWP**: Pod weight plant^{-1}, **PL**: Pod length, **NSP**: Number of seeds pod^{-1}, **100 SW**: 100 seed weight, **GYP**: Grain yield plant-^{1}, **HI**: Harvest index, **GY kg ha^{-1}**: Grain yield kg ha^{-1}

Table 4.4 Descriptive statistics of 14 morphological traits (qualitative and quantitative) traits of mungbean genotypes

Traits	Mean	Range		STD	CV (%)
		Min	Max		
Days to flowering	58.3	41.0	84.0	13.4	23.0
Days to 50% flowering	79.6	54.0	97.0	14.8	18.6
Days to maturity	105.2	71.0	119.0	15.8	15.0
Plant height (cm)	34.6	24.7	51.0	8.3	23.9
Peduncle length (cm)	5.8	2.7	12.5	2.6	45.4
Number of pod peduncle^{-1}	3.9	3.0	5.0	0.9	23.0
Number of pods plant^{-1}	23.1	12.0	43.0	11.3	50.1
Pod weight plant^{-1}	6.6	5.8	13.5	1.7	39.3
Pod length (cm)	9.5	5.7	9.2	1.3	28.2
Number of seeds pod^{-1}	9.3	8.0	11.0	1.4	14.6
100 seed weight (g)	5.3	4.4	6.2	0.7	13.7
Grain yield plant (g)	2.0	3.6	7.6	0.8	41.2
Grain yield kg ha^{-1}	180.7	71.9	290.8	84.8	46.9
Harvest index (%)	15.9	5.0	40.0	7.3	45.7

4.3 Correlations among the quantitative morphological traits

Pearson's correlation coefficient analysis was conducted to identify the relationship among morphological traits (Table 4.5). In general, significant correlations were positive or negative depending on the parameters tested. The number of days to flowering had moderate significant positive correlations with days to maturity (0.56**), plant height (0.54**), and peduncle length (0.58**) and a strong significant positive association with number of days to 50% flowering (0.71*). Similarly, pod weight per plant exhibited a significant positive correlation with plant height (0.51**) and number of pods peduncle^{-1} (0.73*). Harvest index had moderate significant positive correlation with grain yield ha^{-1} (0.62**) and weak significant positive correlation with grain yield plant^{-1}. A high positive significant variation was also observed between pod length and peduncle length (0.80*). Significant and negative correlations were observed between several characters. Harvest index had weak significant negative correlations with days to maturity (-0.56**) and number of pods plant^{-1} (-0.47**).

Table 4.5 Pearson's correlation coefficient among the quantitative morphological traits of mungbean.

Trait	DFL	D 50%FL	DM	PH	PDL	NPP_d	NPP_t	PWP	PL	NSP	100 SW	GYP	HI	GY kg ha^{-1}
DFL														
D 50%FL	0.71*													
DM	0.56**	0.54**												
PH	0.54**	0.43**	0.72*											
PDL	0.58**	-0.67**	-0.58**	-0.52**										
NPP_d	0.18	-0.02	0.23	0.34	-0.18									
NPP_t	0.16	-0.03	0.3	0.51*	-0.26	0.73*								
PWP	-0.17	0.22	-0.17	-0.07	0.09	0.46*	0.33							
PL	-0.40**	-0.45	-0.35	-0.38	0.80*	-0.2	-0.26	0.13						
NSP	0.11	-0.2	-0.19	-0.2	0.13	0.17	-0.08	0.07	0.29					
100 SW	0.03	-0.25	-0.23	-0.01	0.17	0.22	0.09	0.51**	0.09	-0.01				
GYP	0.04	-0.17	-0.13	0.01	0.04	0.11	0.02	0.4	0.15	0.17	0.40			
HI	-0.45*	-0.28	-0.36	-0.38	0.39	0.13	-0.1	0.07	0.11	0.13	0.42	0.42**		
GY kg ha^{-1}	-0.3	-0.24	-0.29	-0.23	0.25	0.12	0.1	0.35	0.09	0.13	0.13	0.40**	0.62**	

**** indicates significant correlation at $P \leq 0.05$ and * significant correlation at $P \leq 0.001$**

DFL: Days to flowering, **D 50%FL**: Days to 50% flowering, **DM**: Days to maturity, **PH**: Plant height, **PDL**: Peduncle length, **NPP_d**: Number of pods peduncle^{-1}, **NPP_t**: Number of pods plant-1, **PWP**: Pod weight plant^{-1}, **PL**: Pod length, **NSP**: Number of seeds pod^{-1}, **100 SW**: 100 seed weight, **GYP**: Grain yield plant-^{1}, **HI**: Harvest index, **GY kg ha^{-1}**: Grain yield kg ha^{-1}

4.4 Morphological cluster analysis

A dendrogram was generated for the eighteen (18) mungbean genotypes based on morphological traits (qualitative and quantitative). A coefficient distance value of 1.23 clustering yielded 4 distinct clusters (Figure 4. 6). The grouping of the clusters, further elaborated in Table 4.6, shows that Cluster I was the largest and comprised 11 genotypes namely: E122, E28, E132, E17, E14, E20, E083, E48, E158, E130 and E092. Out of these genotypes, four (E122, E28, E20 and E083) were collected from the Southern district and three (E158, E132 and E130) from the Central district. This cluster was further subdivided into 6 sub-clusters. Common traits associated with this cluster included: semi erect plants, medium green leaf colour, racemes positioned in the upper canopy, rough testa texture, shortest peduncles, highest number of pods plant^{-1}, least curved pods, and low grain yield plant^{-1} (Table 4.6). The second largest cluster III was identified with five genotypes (E38, E084, Mmelegi, E116 and VC1482E). Two sub clusters were identified within this cluster, one with 3 genotypes (Mmelegi, E116 and VC1482E) while the other sub cluster comprised of E38 and E084. Within the second sub-cluster, genotype E116 and VC1482E were very closely related with a coefficient distance value of almost 0.43, suggesting that these two genotypes were highly similar in their genetic characteristics. The members of this cluster were characterized similarly by racemes positioned throughout the canopy, shortest number of days to flowering, tallest plants, tallest peduncle length, smooth to rough testa texture, largest harvest index as well as highest grain yield per hectare. Mmelegi is a released variety from the Department of Agricultural Research and genotype E116 was originally from the Central District while passport data or information for genotype VC1482E is unknown. The remaining 2 clusters (II and IV) were identified with only one genotype each (TM0117 and E115) and they were collected

from the Southern and Central districts respectively. Cluster II was characterised by distinct black seed colour, long days to flowering and tallest plants and while cluster IV was characterised by tan pod colour, lowest grain yield ha^{-1} and tallest plants.

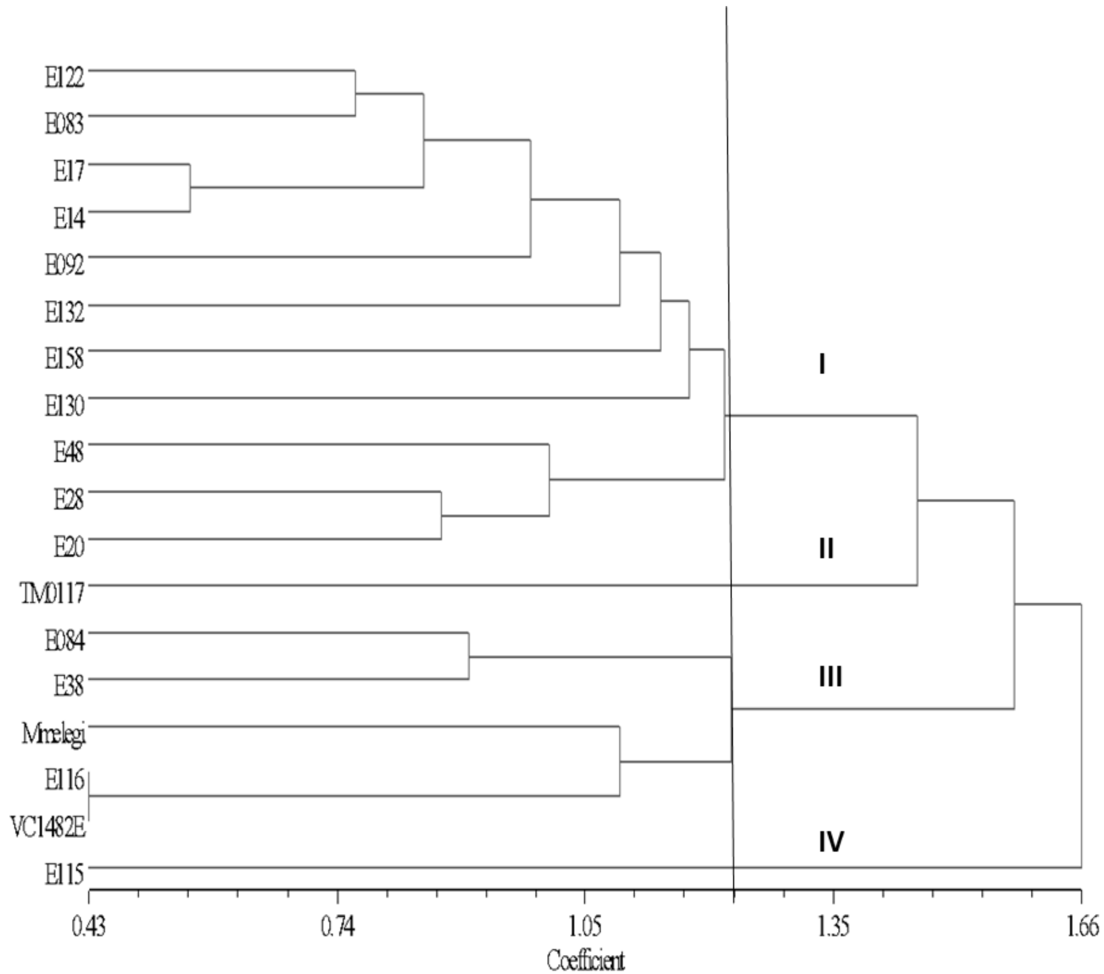

Figure 4.6 Hierarchical clustering using the distance coefficient. The y-axis shows the 18 genotypes and the 4 clusters; x-axis indicates the distance coefficient between clusters. 4 clusters at coefficient approximately (1.23) are shown. The test association; matrix correlation on NTSYS pc (r=0.82).

Table 4.6 Cluster identification and membership of mungbean genotypes

Cluster	Number of genotypes	Genotypes	Cluster identification
I	11	E122 E083 E17 E092 E14 E132 E130 E158 E48 E20 E28	Semi erect, medium green leaf colour, racemes positioned in the upper canopy, shortest peduncles, highest number of pods plant^{-1}, least curved pods, lowest grain yield plant^{-1}, rough testa texture
II	1	TM0117	Long days to flowering, tallest plants, black seeds.
III	5	E084 E38 Mmelegi E116 VC1482E	Short days to flowering and maturity, shortest plants, longest pods, longest peduncles, largest harvest index, highest grain yield ha^{-1} smooth to rough testa texture.
IV	1	E115	Tallest plants, tan pod colour, lowest grain yield ha-1

4.5 Principal component analysis (PCA)

A principal component analysis was applied to show the relationship between the studied traits of the genotypes and extracted 24 principal components (pcs). This PCA was performed on the mean values recorded on 21 morphological traits. The first four principal components which have eigenvalues greater than 1.5 and variable loadings (positive or negative), more than 0.05 were selected as the threshold (Table 4.7). These principal components accounted for 66.7% of the total variability among the 18 genotypes while remaining components contributed only 33.3% of total variability for this set of genotypes with eigenvalues less than 1. The first principal component (PC1) accounted for maximum variability (31.7%) with an eigenvalue of 7.62. The highest positive loading (0.9505) was associated with days to maturity, followed by testa texture (0.9425), days to flowering (0.8659), days to 50% flowering (0.8544), plant height (0.7814) and number of pods per plant (0.63.75), whilst peduncle length (-0.8636) and pod length (-0.7811) had maximum negative loading. The second principal component (PC2) explained 16.6% of the total variation and traits with the highest positive loadings were number of pods per peduncle (0.7331), pod weight (0.7252), number of seeds per pod (0.6123) and pod curvature (0.5328). The third principal component (PC3) had 2.36 as its eigenvalue and accounted for 9.8% of the total variation with the highest positive and negative loadings observed in seed colour (0.5170) and pod colour (-0.5406), respectively. The fourth PC, which explained 8.5% of the total variation, had an eigenvalue of 2.04.

Table 4.7 Principal components, matrix of eigenvalues of the quantitative morphological traits of mungbean

Trait	PC1	PC2	PC3	PC4
Days to flowering	0.8659*	0.0194	0.3336	0.0439
Days to 50% flowering	0.8544*	0.004	0.1224	0.2921
Days to maturity	0.9405*	-0.0863	0.0681	0.1017
Plant height (cm)	0.7814*	-0.2551	0.2988	0.243
Peduncle length (cm)	-0.8636*	-0.0348	0.0155	-0.4408
Number of pods peduncle^{-1}	-0.0499	0.7331*	0.2953	0.1117
Number of pods plant^{-1}	0.6375*	0.2393	-0.1687	0.0067
Pod weight plant^{-1} (g)	0.2534	0.7252*	0.265	0.1219
Pod length (cm)	-0.7811*	-0.058	-0.1224	-0.426
Number of seeds pod^{-1}	-0.3261	0.6123*	0.1491	0.3493
100 seed weight (g)	-0.2815	-0.3516	0.3516	0.0678
Grain yield plant^{-1} (g)	-0.3746	0.5115*	0.1053	0.4884
Harvest index (%)	-0.621*	-0.3298*	0.4906*	0.1084
Grain yield kg /ha	-0.7098*	0.4528	0.1208	0.3301
Growth habit	0.1701	0.2386	-0.38	0.438
Leaf colour	0.0624	0.0624	0.4188	-0.1993
Raceme position	-0.2225	-0.1745	0.3798	0.041
Pod curvature	-0.285	0.5328*	0.427	-0.3928
Pod colour	0.0615	0.1824	-0.5406*	-0.0511
Testa texture	0.9425*	0.0495	0.1999	-0.13
Seed colour	0.3453	0.1311	0.517*	-0.4581
Eigen value	7.62	3.99	2.36	2.04
% of variance	31.7	16.6	9.8	8.5
Cumulative %	31.7	48.3	58.2	66.7

4.6 Shoot, root and nodule traits

The mungbean genotypes differed significantly ($P \leq 0.05$) in nodulation, shoot and root characteristics (Table 4.8). The genotype E158 had the highest mean nodule number (31). As a result of its significantly higher number of nodules, it also yielded a higher nodule dry weight per plant (1.16) compared to other genotypes. Mmelegi produced relatively fewer nodules per plant (7). No statistical differences were found between the genotypes E38, E083, E132, E115, E092, E14, E122 and E20 for the nodule dry weight, but the lowest nodule dry weight was recorded in Mmelegi (0.14 g). All the genotypes produced pink inner nodule colour, which was an indication of efficient nitrogen fixation except for E115 and TM0117 which had a brown colour.

Genotype E083 had the highest shoot dry weight (4.2 g), and the lowest shoot dry weights were associated with Mmelegi and VC1482E at 2.2 g each. Although E083 had the highest shoot dry weight, it was not significantly different from most of the genotypes. Similarly, Mmelegi and VC1482E were not significantly different from the rest of genotypes with lower shoot dry weight values. Root dry weight ranged between 0.4g and 1.4g plant^{-1} with genotype TM0117 recording the highest weight and E084 recorded the lowest root dry weight.

Table 4.8 Variation in nodulation (number of root nodules, nodule dry weight, nodule colour) and shoot and root dry weight of different mungbean genotypes.

Genotype	**Nodule number plant $^{-1}$g**	**Nodule dry weight plant $^{-1}$g**	**Nodule colour**	**Shoot dry weight plant $^{-1}$g**
E38	13^{defg}	0.36^{bcde}	Pink	2.4^{bc}
E116	16^{bcdefg}	0.65^{bc}	Pink	2.4^{bc64}
TM0117	7^{fg}	0.20^{ed}	Brown	2.3^{bc}
E083	14^{bcdefg}	0.33^{bcde}	Pink	4.2^{a}
E132	13^{defg}	0.40^{bcde}	Pink	3.7^{abc}
E17	11^{defg}	0.19^{de}	Pink	3.4^{abc}
E115	16^{bcdefg}	0.44^{bcde}	Brown	3.7^{abc}
E092	17^{bde}	0.45^{bcde}	Pink	3.3^{abc}
E130	16^{bcdefg}	0.30^{cde}	Pink	3.3^{abc}
E14	17^{bde}	0.48^{bcde}	Pink	3.5^{abc}
VC1482E	$9e^{fg}$	0.22^{cde}	Pink	2.2^{c}
E122	19^{cbd}	0.48^{bcde}	Pink	3.3^{abc}
Mmelegi	7^{g}	0.14^{e}	Pink	2.2^{c}
E084	17^{bcde}	0.62^{bcd}	Pink	2.3^{bc}
E20	22^{abc}	0.52^{bcde}	Pink	3.5^{abc}
E28	24^{ab}	0.57^{cde}	Pink	3.6^{abc}
E158	31^{a}	1.16^{a}	Pink	3.2^{abc}
E48	16^{bcdefg}	0.73^{ab}	Pink	3.3^{abc}

Table 4.9 Pearson's correlation among shoot, root and nodule traits of mungbean genotypes

Trait	**NRN**	**NDW**	**SDW**	**RDW**
NRN				
NDW	0.74*			
SDW	0.39**	0.14 ns		
RDW	0.02 ns	-0.07 ns	0.26**	

*= P ≤ 0.05, ** = P ≤ 0.001 and **ns** = non-significant

NRN; Number of root nodules plant^{-1}, **NDW**; Nodule dry weight plant^{-1}, **SDW;** Shoot dry weight plant^{-1} and **RDW**; Root dry weight plant^{-1}

4.6.1 Relationships among the shoot, root and nodule traits of mungbean

Pearson's correlation coefficients revealed both positive and negative significant associations among the evaluated shoot, root and nodule traits (Table 4.9). A strong positive significant association (r= 0.74) was observed between number of root nodules and nodule dry weight. Similarly, there was a weak significant positive correlation between number of root nodule and shoot dry weight with corresponding correlation coefficient of (r = 0.39). A non-significant positive correlation was observed between number of root nodules and root nodule dry weight (r = 0.02), and nodule dry weight and shoot dry weight (r = 0.14). The results also revealed weak significant association between root dry weight and shoot dry weight (r = 0.26). A weak negative

non-significant correlation (r = -0.07) was observed between nodule dry weight and root dry weight.

4.7 Photosynthetic traits

Transpiration rate (E), net photosynthetic rate (Pn), stomatal conductance (Gs), and chlorophyll content were significantly different ($P \leq 0.05$) among the mungbean germplasm studied (Table 4.10). Higher values of transpiration rate (E) were observed in TM0117 (5.6 mmol H_20 m^{-2} s^{-1}). E083 recorded the lowest value (3.3 mmol H_20 m^{-2} s^{-1}), while the rest of the genotypes were not significantly different from each other. With regard to net photosynthetic rate, VC1482E had a higher value (15.4) while E28 and E084 maintained relatively lower values of 9.30 µmol $C0_2$ m^{-2} s^{-1} and 9.60 µmol $C0_2$ m^{-2} s^{-1}, respectively. Statistically, VC1482E was different from E28 and E084 but not significantly different to the rest of the genotypes. Genotype E116 had higher stomatal conductance of 245 mmol m^{-2} s^{-1} than the other genotypes, while TM0117 exhibited lower stomatal conductance of 64.3 mmol m^{-2} s^{-1}. Chlorophyll values ranged between 47.4 and 56.4 with the highest value recorded in genotype E17. However, no significant differences were observed in most of the genotypes except for the genotype E132 which exhibited a significantly lower chlorophyll content.

Table 4.10 Variation in mean performance of photosynthetic parameters of mungbean

Genotype	Transpiration rate (E)mmol $H_2Om^{-2}S^{-1}$	Net photosynthesis (Pn) µmol $CO_2m^{-2}S^{-1}$	Stomatal conductance (Gs) mmol $m^{-2}S^{-1}$	Chlorophyll (spad reading)
E38	4.57^{abc}	11.97^{abc}	95.6^{cd}	48.36^{ab}
E116	3.5^{bc}	11.7^{abc}	245.0^{a}	51.0^{ab}
TM0117	5.6^{a}	10.9^{bc}	64.3^{d}	55.2^{ab}
E083	3.3^{c}	11.2^{abc}	82.2^{ab}	49.6^{ab}
E132	4.9^{abc}	11.3^{abc}	129.2^{abcd}	47.43^{b}
E17	4.4^{abc}	12.57^{abc}	113.1^{bcd}	56.4^{a}
E115	4.2^{abc}	11.2^{abc}	86.5^{cd}	50.87^{ab}
E092	4.77^{abc}	13.5^{abc}	128.1^{abcd}	53.5^{ab}
E130	4.57^{abc}	14.87^{ab}	91.5^{cd}	51.3^{ab}
E14	5.1^{ab}	10.6^{bc}	98.1^{cd}	55.5^{ab}
VC1482E	4.57^{abc}	15.4^{a}	225.3^{ab}	49.03^{ab}
E122	5.3^{ab}	14.1^{ab}	100.6^{bcd}	52.23^{ab}
Mmelegi	5.03^{abc}	10.7^{bc}	196.67^{abc}	52.83^{ab}
E084	3.97^{abc}	9.57^{c}	183.4^{abcd}	49.0^{ab}
E20	4.83^{abc}	12.4^{abc}	121.45^{abcd}	48.03^{ab}
E28	4.5^{abc}	9.27^{c}	147.3^{abcd}	52.9^{ab}
E158	4.13^{abc}	10.53^{bc}	84.9^{cd}	51.9^{ab}
E48	3.7^{bc}	12.57^{abc}	104.6^{bcd}	50.06^{ab}

[a]Values followed by different letters in the same column indicate significant differences at $P \leq 0.05$ according to LSD test

4.8 Molecular characterization

4.8.1 Polymorphism of SSR primers among the mungbean genotypes

Out of the 44 SSR markers that were initially screened, fifteen produced clear amplified products and these were further used to evaluate molecular diversity of the 18 mungbean genotypes. The results of analysis are presented in Table 4.11. A total of 167 bands were generated by the fifteen SSR primer pairs analysed in this study. All the bands produced were polymorphic in nature with alleles assigned based on different fragment sizes (base pair). The scored fragment sizes ranged from 100 to 300 base pairs (bp) in length. The maximum number of amplified bands (23) was produced by primer LR7323B, and the minimum (1) was observed in primer CEDG305 and DMB-SSR 182**.** Figures 4.7 and 4.8 show gel images of amplified loci which yielded the highest number polymorphic bands. Polymorphic information content (PIC) values ranged between 0.23 and 0.99. CEDG305 and DMB-SSR 182 exhibited the highest PIC value (0.99), while LR7323B had the lowest value (0.23) among all SSR primers. The number of alleles per locus ranged from one to three among the mungbean genotypes. Nine polymorphic primers had 1 allele each, 4 detected 2 alleles while remaining 2 primers had 3 alleles each. The highest allele number (3) was revealed by primers LR7323B and CEDG264.

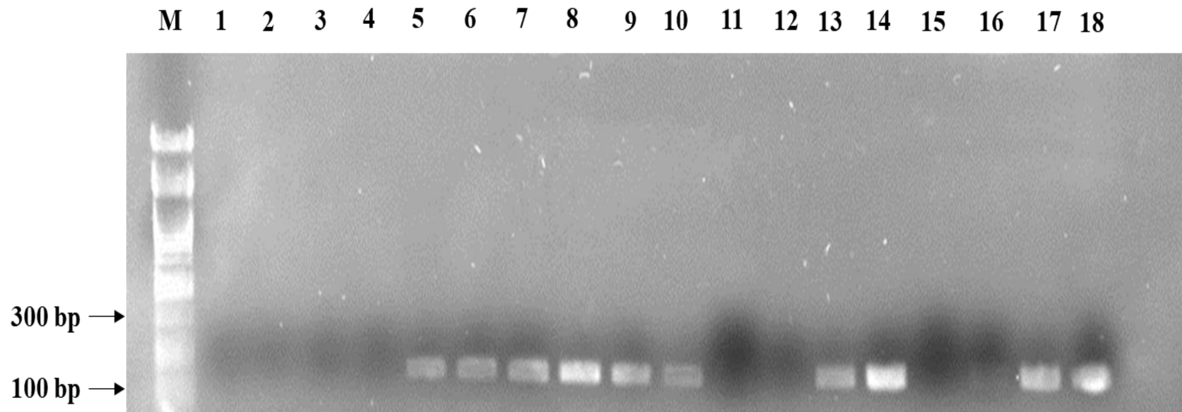

Figure 4.7 Gel picture showing polymorphic bands using primer GBssr-MB77, numbers 1-18 represent the mungbean genotypes, M- 1kb DNA ladder

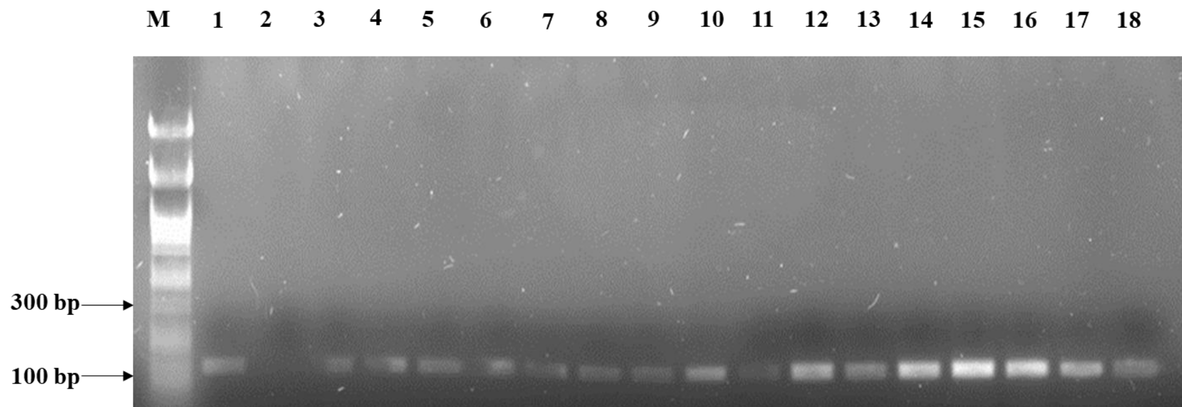

Figure 4.8 Gel picture showing polymorphic bands using primer CEDG056, numbers 1-18 represent the mungbean genotypes, M- 1kb DNA ladder

Table 4.11 List of amplified SSR primers, number of polymorphic bands, number of alleles and polymorphic information content (PIC) values

SSR Primer	No of polymorphic bands	No of alleles per loci	PIC value
LR7322B	12	1	0.55
LR7323A	12	1	0.55
LR7323B	23	3	0.23
GBssr- MB77	10	1	0.69
VR 188	13	2	0.74
VR 225	10	1	0.69
Vr SSR 13	8	1	0.74
Vr SSR 17	19	2	0.69
CEDG006	13	1	0.8
CEDG092	9	2	0.43
CEDG305	1	1	0.48
CEDG264	12	3	0.8
LR7338A	6	1	0.99
CEDG056	18	2	0.84
DMB-SSR	1	1	0.89

4.8.2 Cluster analysis based on SSR markers

A dendrogram was constructed to determine the genetic diversity and relatedness among eighteen (18) mungbean genotypes based on SSR analysis by the unweighted pair group method with arithmetic means (UPGMA) method with NTSYS pc 2.1 program. UPGMA cluster analysis grouped the genotypes into seven distinct groups formed at a coefficient distance value of 1.16 (Figure 4.6) with values ranging from 0.41 to 1.91. The number of genotypes per cluster varied

from one genotype in cluster VIII, to five genotypes in cluster I. The first main cluster (I) was composed of genotypes E116, E084, VC1482E, E083 and E122. This cluster was further subdivided into four sub clusters revealing sufficient amount of diversity within the cluster. The second main cluster (II) consisted of 4 genotypes, two from the Southern District (E20 & E28). The SSR marker dendrogram used could not distinguish between E14 and E132 from Southeast and Central Districts respectively, while E20 was distinct from the rest of the genotypes within this cluster. Cluster V consisted of a mixture of two genotypes from Central District, together with one from Gantsi and Kgalagadi Districts, respectively. The remaining clusters namely: III, VI and VII had only one genotype each, being E158, E17 and E092 respectively. The genotype E092 was separated from all other genotypes analysed at a coefficient distance of 1.91 and appeared as the most divergent genotype, indicating that it is genetically dissimilar from the other genotypes. This demonstrates the distinctiveness of the genetic background of this genotype from all the others.

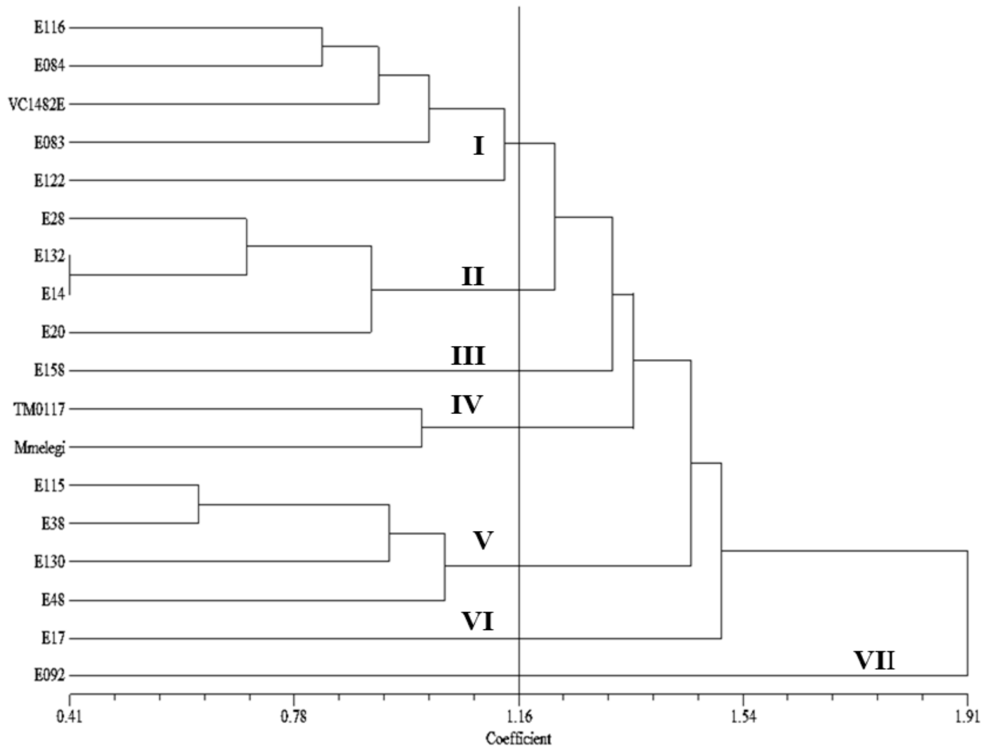

Figure 4.*9* Dendogram showing genetic similarity of 18 mungbean genotypes revealed by UPGMA cluster analysis based on SSR markers. Seven clusters were observed at coefficient of 1.16

CHAPTER 5

5.0 DISCUSSIONS

5.1 Morphological diversity

5.1.1 Qualitative morphological variation

Assessment of genetic diversity and relationships among crop germplasms is a critical step for improvement of agricultural crops. However, proper use of genetic diversity within germplasm requires a good knowledge about their characteristics and in that instant morphological traits have been used as a valuable guide for studying genetic variation in crop plants, which is of great interest to plant breeders (Mohammadi and Prasanna, 2003).

The results of this study revealed the existence of qualitative morphological variation among the mungbean genotypes evaluated on the basis of frequency distribution, indicating genetic divergence across the genotypes. A substantial amount of variability was exhibited in the vegetative growth parameters assessed, which included growth, habit, leaf colour, raceme position. However, variation did not exist for growth pattern, terminal leaflet shape and flower colour characteristics. Similarly, with respect to pod and seed related parameters, variation was evidenced in most of the traits except seed shape. Genetic diversity among various crop species using morphological traits such as vegetative growth traits, leaf traits, pod and seed traits, and yield have been carried out by researchers on various crop species (Elteib and Gasim, 2020 ; Adewale *et al*., 2012) and all these traits were found to be of great importance in assessing genetic variation. In this respect, the results of this study are consistent with those of Doumbia *et al*. (2013)who reported variation in most of the qualitative characters studied in cowpeas. Raceme position is one of the

traits that could be considered to be important because varieties with racemes above the canopy are considered easier to harvest and require less labour than those with raceme below canopy (Bennett and Ofori, 1999). In our study, 67% of genotypes had their racemes above the canopy thus making them genotypes of importance when the raceme position is considered for selection. Moderate seed colour variation was observed among the tested genotypes with the green colour (94%) being the most common and the remaining 6% associated with the black colour (TM0117). Findings reported by Al-saady *et al.* (2018)who studied diversity in mungbean germplasm in respect of seed traits also revealed that from the 25 accessions evaluated, 4 groups of seed colours were identified with the largest group predominated by the green colour which is in line with our present findings. Similar findings were also reported by Wang *et al.* (2018) where green seeds were more recurrent (78%) than black seeds (13%) in mungbean landraces of China. The assessment of the same character was slightly different from Tripathy *et al*. (2016) who found a wide range of seed colour variation. In this study, green was the predominant seed colour and according to previous studies, farmers and consumers preferred green coloured varieties over brown and black seeded; this made the green types more easily accepted in food markets when compared to other seed colours (Mbeyagala *et al*., 2017b; Karimi *et al*., 2019). The differences observed in these morphological trait were not influenced by geographic origin.as some genotypes from the same location had varying colours. Overall, qualitative morphological characters including growth pattern, flower colour, terminal leaflet shape showed no variation among the evaluated mungbean genotypes. The most probable explanation for the absence of variation in these traits could be that the genes controlling these characters were dominant in all the genotypes hence, not beneficial to consider them during selection for improvement of mungbean.

5.1.2 Quantitative morphological variation

Observations into the quantitative morphological data revealed a significant ($P \leq 0.05$) variation in the fourteen quantitative traits assessed indicating the presence of sufficient amount of genetic variability among the mungbean genotypes that could be harnessed for crop improvement (Table 4.3). The earliest flowering was observed in genotypes VC1482E, E084, E38 and E116 and the maximum number of days taken to flower was observed in genotype TM0117 and E28. The range of days to flowering obtained in this study was higher than the values reported by Patel *et al*. (2019) who observed that days to flowering ranged between 37 and 63 days among the 44 mungbean accessions evaluated. Early flowering in genotypes implies early physiological maturity therefore, this early maturity trait could be used as an effective drought escape mechanism (Shavrukov *et al*., 2017), a desirable character in breeding for drought tolerance in mungbean. Moreover, farmers are more interested in early maturing genotypes for improved crop yields. Therefore, this early flowering genotypes (VC1482E, E084, E38 and E116) can be selected for early maturity. Significant variation was also observed in peduncle length with values ranging between 3.3 and 10.13 cm. A similar range was observed by Ahmad and Belwal (2020) who also reported variation in peduncle length with values ranging from 4.10 to 10.32cm, with values among individual genotypes varying significantly. In comparison to Mmelegi, E116 and E084 had the highest peduncle length values, but there was no statistical significance from this outcome and collection sites had no effect on variations in peduncle length as it was displayed across different villages. The longer peduncles have shown a close positive association with the pods position in this study thus this suggest that genotypes with longer peduncles could be considered during selection of parental genotypes as it influences the position of the pods, thus easing visibility of

pods during harvesting. The highest coefficient variation was observed in number of pods per plant and similar observations have been reported by Win *et a*l. (2020) and this can be considered as highly variable trait for the data set. Grain yield per hectare displayed variation among evaluated genotypes ranging from 71.9 kg ha^{-1} (E115) to 290.8 kg ha^{-1} (E116). E116 was the highest yielding genotype and possible reasons for this could be due to it being the earliest flowering and maturing genotype, has longest pods and peduncles and also characterized by short plants. On contrary, E115 was the poorest yielder and lower grain yield exhibited by this genotype could be due to low number of seeds pod^{-1} and lowest grain yield $plant^{-1}$. From this study, it has been observed that most mungbean genotypes with shorter plants tend to yield better than those with taller plants. Wang *et al*. (2018) described plant height as a fundamental morphological character that is highly predictive of biomass and directly related to yield potential of various crops. With respect to yield (kg ha^{-1}), the absence of statistical difference between Mmelegi and other genotypes emphasizes the similarity of genotypes to the control. Again, the results did not show any distinct association between grain yield and collection site. One possible explanation for this observation may be due to differences in genetic characteristics of the evaluated genotypes. Overall, the results of this study based on the quantitative morphological traits are supported by previous works of (Payasi, 2015; Shyamalee *et al*., 2016; Tripathy *et al*., 2016; Patel *et al*., 2019; Win *et al*., 2020) proving the existence of variation in morphological traits among mungbean genotypes thus indicating the potential for genetic improvement for traits of interest. Therefore, selection of genotypes based on these traits would be effective for the improvement of this crop, and also, for broadening its genetic base.

5.1.3 Relationship among the quantitative morphological traits

Establishing the extent of relationships of various quantitative characters is very crucial for varietal selection and for a successful breeding programme (Sarkar *et al*., 2014). Correlation studies provide detailed information to identify suitable and superior characters for efficient yield improvement and, also, determines traits influencing variation and interrelatedness when selecting desirable parental lines towards yield improvement. The correlation results from this were not partitioned to associations among the genotypes but rather as a result of relationships among the evaluated traits. Number of days to flowering showed a significant positive correlation with days to maturity. Similarly, Shyamalee *et al*. (2016) and Singh and Kumar (2014) observed significant correlation between these two characters suggesting that these traits are heritable and can be transferred into desired genotypes. Singh and Kumar (2014) specified that flowering time could also be an important indicator of maturity. In our study, the relationship between harvest index and number of pods per peduncle was also significant and this is in agreement with Hozayn *et al*. (2013) who studied genetic variability in 16 exotic mungbean genotypes for late sowing under Egyptian conditions and found that harvest index was positively correlated with number of pods per plant, and this implies that the association among these characters shows that harvest index is equally important in determining the number of pods a plant can bear, thus making it an important trait to consider when selecting for yield. From the correlation results, it is evident that traits such as number of pods per plant, harvest index, days to flowering and days to maturity are essential and justifiable related to yield (Table 4.5); this thus makes them important traits to be considered when developing an effective reliable selection index for the development of promising mungbean genotypes.

5.1.4 Cluster analysis of morphological traits

To obtain an idea about the extent of the similarities and differences among the germplasms based on the morphological qualitative and quantitative traits, cluster analysis was performed using UPGMA (unweighted pair group method with arithmetic mean). Cluster analysis is essential in identifying accessions with useful traits belonging to distinct clusters for hybridization (Soe *et al*., 2019). Based on dendrogram, mungbean genotypes were successfully grouped into four (4) main clusters. This finding is comparable with the results of (Hapasari *et al*., 2018) who studied diversity of 122 Indonesian germplasm and the dendrogram clustered these into four groups. From this study, it was observed that the genotypes within larger groups, were distantly related based on their collection sites or geographic origin, indicating that there is no association between clustering pattern and geographic distribution. However, the results of this study revealed that grouping of genotypes was based on shared similar morphological characteristics e.g. genotypes of cluster III, which was composed of Mmelegi, E084, E38, E116 and VC1482E, were grouped together on the basis of lowest number of days to flowering, tallest plant, longest peduncle, largest harvest index as well as highest grain yield per hectare. Additionally, the clustering pattern also revealed that genotype TM 0117 (cluster II) and E115 (cluster V) were placed separately from other genotypes, and they should be given attention during selection as they are considered to be superior than other genotypes based on black seed colour and tan pod colour respectively.

The results of this study concur with the study of Mohan *et a*l., (2019)who reported genetic diversity among the mungbean genotypes and clustering was based on different morphological traits. These results were also supported by findings of Win *et al*. (2020) who assessed genetic diversity based on morphological and agronomic characters among 185 mungbean accessions by

multivariate analysis and the genotypes in their study were grouped into four clusters based on agro-morphological traits. Although the studied germplasm was collected from different villages in Botswana, they shared some morphological traits suggesting that in this present study the clustering did not always reflect the geographic origin of the accessions. Lang *et al*. (2009) reported that although the varieties come from different places, they can be grouped together because of close similarities in terms of qualitative traits; in contrast Rohman *et al*. (2004) reported that genetic diversity is generally associated with geographical diversity. Therefore, it can be suggested that selection of genotypes based on characters present in these clusters can be used as possible parental combinations carrying the desirable traits that could be exploited for further mungbean genetic improvement. Genotypes from cluster I and III could also be utilized as potential parents for development of high yielding varieties as they were mostly characterized by high yield related traits (Figure 4.3).

5.1.5 Qualitative and quantitative traits principal component analysis

Principal component analysis was employed on seven quantitative and fourteen qualitative traits to identify characters that played principal roles in diversity of the evaluated mungbean genotypes. According to Khodadadi *et al*. (2014) principal component analysis is a multivariate technique that explores complex data sets and transforms a number of associated variables into a smaller number of PCs. It assists plant breeders to identify potential parents for hybridization and identify characters that contribute high variation among the genotypes studied (Doumbia *et al*., 2013; Soe *et al*., 2019). From the present study, the PC analysis partitioned the total variance into 4 PCs contributing maximum (67%) to the total variation among tested genotypes. These PCs had eigenvalues greater than 1.5. This implies that these genotypes greatly varied in most of the traits

evaluated. The main quantitative characters which accounted for most variability in PC1 included days to flowering, days to 50% flowering, days to maturity and plant height, but testa texture was the quantitative character that contributed most positively compared to the rest of the traits. On the other hand, peduncle length, pod length, harvest index and grain yield per hectare contributed more negatively than other characters in this PC, suggesting that they played a minor role in the genetic variation of the studied mungbean genotypes. This finding is in consonance with those of Waniale *et al*. (2014) whose results revealed that days to flowering, days to maturity and plant height were among the characters that contributed largely to variability. These results were also comparable with the findings of (Singh *et al*., 2014) who found three PCs accounting for most (74%) of the variation in mungbean landraces and characters contributing more positively to PC1 including; plant height, days to flowering, number of pods per plant and number of seeds per pod.

The second PCA was more related to pod/seed traits and yield contributing traits such as number of pods per peduncle, pod weight per plant, number of seeds per pod, grain yield per plant and pod curvature. These results partly agree with the findings of Thippani *et al*. (2017) who reported that traits that contributed positively with respect to the third PC were pod colour and seed colour. In many genetic diversity studies, it has been discovered that the first three components are the most suitable for revealing variations among various crop species (Evgenidis *et al*., 2011; Bhanupriya *et al*., 2014; Akpertey *et al*., 2019; Al-Naggar *et al*., 2020). From these findings, it can be suggested that the key characters with the largest vector loadings which include days to flowering, days to 50% flowering, days to maturity, plant height, pod weight per plant, number of pods per plant, number of pods per peduncle, number of seeds per pod, grain yield per plant, grain yield per hectare testa texture, pod curvature and seed colour were the most discriminating characters explaining

greater variability in mungbean genotypes. Based on these key characters, genotype E116, E084 and E122 (Table 4.7) outperformed all the other genotypes including our control (Mmelegi) hence they should be given more emphasis when identifying potential parental materials aimed at mungbean improvement.

5.2 Variation in shoot root and nodule traits

The results of this study showed that mungbean genotypes varied significantly ($P \leq 0.05$) in nodulation, shoot and root characteristics (Table 4.8). The high nodule dry matter recorded by E158 reflects a more efficient symbiotic nitrogen fixation that could result in an increased shoot biomass. Interestingly, the highest nodule number and nodule dry weight were also recorded in genotype E158 and the differences between the nodule dry weight per plant may be attributed to the number of the nodules produced by the genotype. With regard to nodule colour, most of the genotypes in this experiment exhibited pink inner tissues, indicating the presence of iron containing protein essential for effective nitrogen fixation (Farid and Navabi, 2015). Pink nodules are known to contain and actively express dinitrogenase reductase (nifH) genes that codes for the synthesis of nitrogenase enzymes responsible for the reduction of N_2 to NH_3 (Gaby and Buckley, 2014). TM0117 and E115 produced nodules brown in colour, which is an indication of nodule ineffectiveness. According to Van de Velde *et al*. (2006) the presence of the brown/green colour is due to the degradation of the heme group associated with leghaemoglobin. The number of nodules positively correlated with shoot dry weight indicating the reliance of shoot biomass on nodulation (Kawaka *et al.*, 2014). The correlation analysis showed a significant relationship between shoot dry weight and root dry weight (Table 4.9). These results are consistent with the observation made by Kawaka *et al*. (2014) who found significant positive correlation between

shoot dry weight and root dry weight together with number of root nodules and nodule dry weight in common bean suggesting these variables could be regarded as reliable indicators for effective nitrogen fixation.

5.3 Variation in photosynthetic parameters

Photosynthesis is the key physiological processes for the production of plant assimilates (Zakariyya and Prawoto, 2015). In the present study, photosynthetic rates (Pn) were measured in addition to other surrogates like stomatal conductance (gs), transpiration rate (E), and chlorophyll content (SPAD). Transpiration rate varied significantly between 3.3 mmol H_20 m^{-2} s^{-1} and 5.6 mmol H_20 m^{-2} s^{-1} with the highest value recorded in genotype TM0117 and lowest in E083. Since E083 had the lowest transpiration rate values, it might be able to avoid water stress during the growing period. According to Brodribb and Holbrook (2003) transpiration is associated with the regulation of stomatal opening thus variations in stomatal opening causes changes in water potential. Zakariyya and Prawoto (2015) regarded transpiration rate as an important physiological activity that plays a role in stimulating the rate of absorption and transport of water and nutrients, keeping plant cells turgidity to remain in optimum condition and maintain the stability of leaf temperature. Similarly, significant differences ($P \leq 0.05$) in net photosynthesis were found among the mungbean genotypes. When compared to other genotypes, VC1482E had higher photosynthetic values but differed significantly from E084 and E28. The highest photosynthetic rate values associated with VC1482E might be inherent in this genotype. However, the results of this study clearly showed that genotypes with the highest photosynthetic rates were not necessarily the highest yielders and vice versa. Therefore, other environmental factors not clearly examined in this study could have an influence on crop yields rather than rate of photosynthesis *per se*.

Stomata are tiny pores on a leaf surface that regulate gas exchange by facilitating the diffusion of carbon dioxide (CO2) and preventing excessive water-loss through transpiration (Haworth *et al*., 2011) and according to Sheshshayee *et al*. (2003) both transpiration and photosynthesis mainly depend on the closing and the opening of the stomata. The results of this study showed that stomatal conductance rates displayed a high range (64.3 - 245 mmol m^{-2} s^{-1}) of variability ($P \leq 0.05$) among the evaluated mungbean genotypes, with E116 and VC1482E displaying maximum values while TM0117 maintained lower values. A plausible explanation for such variation could be attributed to some genotypes developing mechanism which allows closing of stomata in response to water deficit. Low stomatal conductance values observed in TM0117 are likely to be associated with stomatal closure which occurs when the mesophyll begins to dehydrate (Cardona-ayala *et al*., 2013). Another possible reason for stomatal closure which led to low stomatal conductance values could have been due to increased VDP (Vapour Pressure Deficit). When the vapor pressure difference (VPD) between the leaf and the atmosphere increases, guard cells loose turgor, thereby leading to stomatal closure to moderate the impact of evaporative demand on plant water loss (McAdam and Brodrib, 2015; Buckley, 2019). According to Bartlett *et al*. (2016) when water becomes limited, signals such as reduced hydraulic conductivity and abscisic acid (ABA) rise, causing guard cell turgor pressure to decrease, and results in reduced stomatal aperture and stomatal conductance. From the results of this study it can be concluded that when plants sensed unfavourable conditions (water stress and high vapour pressure deficit), they kept their stomata closed most of the time to preserve the available water as much as possible hence lower stomatal conductance values. However, with respect to genotypes which exhibited higher values, it can be suggested that stomata have failed to close or were partially open even when sensing changes in water availability, and the plants did not exhibit any symptoms of wilting. On the other hand,

genotypes with high stomatal conductance could have a greater transpiration potential while at the same time posing a risk of excessive water loss during water shortage (Tombesi *et al*., 2015).In a nutshell, these results suggest the existence of variations in stomatal regulation in the genotypes that could be beneficial when breeding for drought tolerance in mungbean varieties.

Variation in stomatal regulation among mungbean genotypes evidenced from the results of this experiment may vary considerably depending on other factors such as age of the leaf (Mills et al..2008) temperature (Urban et., 2017) and time of data collection number of dry cycles of leaf co. Due to some technical reasons beyond our control, data on stomatal conductance was not collected together with other photosynthetic and yield related traits from the field but from the greenhouse experiment as already stated from the results section. This implies that it's impossible to relate the stomatal conductance values with photosynthetic rate as well as yield because the results that we have, were only limited to the greenhouse experiment, hence addressing them independently, hence stomatal conductance values from the greenhouse experiment cannot be used to extrapolate field values.

The chlorophyll content values ranged between 47.3 and 56.4 suggesting considerable variation in leaf chlorophyll due to genotypic differences. The genotypes were statistically similar except that E132 had higher values than E17. SPAD chlorophyll meter has been widely used as an indirect measure of leaf chlorophyll content, photosynthetic capacity and is a good indicator of leaf N in various crop species (Netto *et al*., 2005; Meena & Massawe, 2013; Islam *et al*., 2014). Therefore, the higher chlorophyll content associated with genotype E17 indicates that the genotype has a better light harvesting mechanism as compared to only E132. With the higher level of chlorophyll content associated with E17 compared to other genotypes, one would expect an increase in light

absorption leading to improved photosynthetic capacity of this genotype. However, higher values of photosynthetic rate and grain yield per plant were exhibited by different genotypes indicating that there was no relationship between chlorophyll content and these traits. Hence, these values might be considered insignificant as they were not adequate enough to improve the photosynthetic potential of this genotype. According to Netto *et al*. (2002) variations in chlorophyll can be influenced by environmental stress in plants together with senescence and damage but because in our study the genotypes were given the same management, this environmental stress factor can be ruled out and then differences attributed to genotypic ones.

5.4 Molecular diversity

5.4.1 Polymorphism and effectiveness of the SSR markers

Simple sequence repeat markers (SSR) have been used to assess genetic diversity of mungbean germplasm and various crop species (Wu and Huang, 2007; Reflinur *et al*., 2016; Molla *et al*., 2016; Kaur *et al*., 2018; Wang *et al*., 2018). From this study, the level of polymorphism detected among the 18 genotypes demonstrated the efficiency of SSR markers for discriminating between distinct genotypes. The results of this study showed that the fifteen SSR primer pairs used, showed scorable polymorphism. The PIC values ranged from 0.23 to 0.99 with an average of 0.67. Twelve of the 15 markers were highly informative, with PIC values ranging from 0.55 and 0.99 and the remaining markers were reasonably informative except for marker (LR7323B), which was slightly informative with PIC value of 0.23. According to Botstein *et al*. (1980) markers with PIC values of ≥ 0.5 in genetic studies are highly informative and effective in distinguishing the polymorphism rate of a primer at a specific locus. These results are in close agreement with the findings of Wang *et al*. (2018) who used 20 SSR markers for the estimation of genetic diversity among 184 mungbean genotypes and PIC values ranged from 0.504 to 0.753. Molla *et al*. (2016) also found PIC values ranging from 0.538 to 0.803 with an average value of 0.637 among 42 mungbean genotypes evaluated using 5 SSR markers. The differences or the higher PIC values reported from this study could have been influenced by a number of factors such as number of SSR loci and the nature of germplasm. Polymorphism Information Content is important as it demonstrates the informativeness of the SSR loci and their potential to reveal differences among genotypes based on their genetic relationships (Legesse *et al*., 2007). The distribution of informative markers is the key attribute in reflecting the overall diversity in the crop populations (Nielsen *et al*., 2014). A

great proportion of polymorphic markers revealed in this study could be beneficial in establishment of inter-specific hybrids and monitoring the flow of desirable genes to promising mungbean genotypes.

The number of alleles amplified per primer pair ranged from 1 to 3 for the evaluated mungbean genotypes. These findings were in line with the number of alleles detected using SSR markers in previous mungbean genetic studies by Gupta *et al*. (2014) and Gwag *et al*. (2006). They identified the number of alleles per SSR locus ranging from 2 to 3 and 2 to 5 respectively, with an average of 2 and 3 alleles per locus. These findings were also comparable to observations made by Gupta *et a*l. (2013) who used 245 SSR primers to amplify genomic DNAs extracted from 24 diverse genotypes of blackgram and the detected number of alleles ranged between 2 and 3. Gupta *et al*. (2014) also reported similar results while assessing genetic variability among twenty mungbean genotypes using twenty-seven SSR markers. However, our results were slightly lower than the results reported by Reflinur *et al*. (2016) who found a range of 3 – 8 alleles per locus in some mungbean genotypes. According to Legesse *et al*. (2007) allelic differences in marker systems have been reported to be influenced by factors such as the methodologies applied for detection of polymorphic makers and, also, the number of SSR loci hence this could be one possible explanation for obtaining such results in our study. The small quantity of alleles detected per locus observed in the present study indicated a considerable magnitude of diversity among the genotypes, suggesting the opportunity to select diverse germplasm and more SSR primers with scorable alleles for possible use in future mungbean breeding programmes. SSR markers were highly informative and polymorphic as evidenced from high PIC values obtained in this study, and this is a good indication of their informative potential and can be used to reveal differences among

mungbean germplasm. Therefore, these SSR markers are highly important and could be used in genotyping and diversity analysis of mungbean germplasm.

5.4.2 Molecular diversity based on cluster analysis

Genetic diversity was analysed using the SSR markers because of their ability to detect high levels of polymorphism at each locus and high discriminating power as noted by Vieira *et al*. (2016). Furthermore, these markers are evenly distributed throughout the plant genome, easily automated, and good analytic resolution made them widely preferred in genetic diversity studies. The SSR-based dendrogram constructed using the UPGMA hierarchical clustering clearly distinguished the genotypes into seven main clusters (Figure 4.6) at similarity coefficient of 1.61 and a fair representation of genotypes from different geographical location was observed in each cluster. Dendrograms based on morphological traits and SSR markers showed differences in total number of clusters and positioning of genotypes within clusters. One possible reason for this variation in clustering might be the environmental influence, that most of the morphological traits are polygenic and greatly affected by environmental factors (Dalamu *et al*., 2012)**.** Furthermore, Dey *et al*. (2006) explained that molecular markers are distributed throughout the genome (coding and non-coding region) and nearly 90% of the regions of the genome are not expressed at phenotypic level, hence very difficult to find out similarity between morphological and SSR marker clustering. In overall, the results of this study showed that clustering pattern in both morphological and molecular analysis revealed that there was no association between geographical distribution and morphological differences nor similarities of the evaluated of genotypes. Clustering of genotypes originating from adjacent districts was also identified in molecular cluster analysis. For example, genotypes E38 from Gantsi and E48 from the Kgalagadi District and this suggests a common

genetic background. Even though grouped together because of their relatively close geographic proximity to one another, in morphological clustering, the two were clustered differently on the basis of their morphological differences. According to Landguth *et al.* (2015) the evolutionary forces such as gene flow, genetic drift, mutation, migration, selection, and germplasms exchange play a role in distributing accessions into different clusters. Grouping of these genotypes based on their morphological differences, suggest that high rates of gene flow might have occurred between genotypes and their geographical areas of collection, probably due to of exchange of seeds between districts through human migration. Clustering analysis based on SSR markers showed a close relationship between genotype E132 and E14, which helped to identify duplicates within the evaluated genotypes, since molecular markers represent a portion of the genome that is not subjected environmental influence (Semagn *et al*., 2006). Furthermore, when associated with morphological clustering, the same genotypes were grouped together from different geographical areas of collection and shared similar morphological characters. Being in a close relation, suggest that the genotypes were unable to be distinguished genetically and have similar genetic backgrounds, and this could have resulted from free exchange of seeds among farmers across different agro ecological zones. Therefore, this finding, suggests that in future mungbean breeding activities, genotypes with close similarities could be represented as one genotype so that only one genotype can be chosen to avoid duplication and avoid crossing amongst themselves.

The results of this study indicated that genotypic variation revealed by clustering of mungbean genotypes through SSR markers could possibly be related to the genetic differentiation among individual genotypes rather than geographical origins and this is supported by the findings of Kanavi *et a*l., (2019); Wang *et al*., (2018); Markam *et al*., (2018) and Bhuyan *et al*., (2014) (RADP

analysis). SSR analysis by Kanavi *et al*. (2019) revealed the existence of eight major groups of genotypes within the 56 evaluated green gram germplasm of which cluster I was more diverse when compared to all other clusters. However, contrary to the findings of this study (Lakhanpaul *et al*., 2000) observed that genetic variation through RADP analysis was due to geographic differentiation i.e. genotypes collected from one locality tended to fall in the same cluster in a dendrogram. Clustering of crop germplasm is more useful in understanding the genetic relatedness and to select suitable genotypes for breeding and conservation strategies. All genotypes were dispersed throughout the UPGMA clusters. According to Barnaud *et al*. (2007) farmers' practices and historical factors affect patterns of genetic diversity. Despite the gene flow, farmers' practices are key to maintenance of genetic diversity of landraces with appropriate agronomic and ecological traits. A close genetic similarity that was observed between E132 and E14 could be attributed to free exchange of seeds among farmers across different agro ecological zones and they may have ended up being named differently. It can be suggested that the two genotypes are the same despite being collected from different locations. Wang *et al*. (2018) suggested that introduced accessions should always maintain their original passport information in both collection and introduction to avoid extra labour in germplasm management. Genotype E092 was quite distant from the other genotypes and distinguished by the following yield and yield contributing traits with minimum values such as harvest index, pod length, grain yield per plant and pod weight per plant together with maximum days to maturity. Carpentieri-pípolo *et al*., (2000) highlighted that the use of divergent parents in hybridization programs is aimed towards producing high heterosis in progenies and to increase the chances of obtaining superior segregants to broaden the genetic base. Yield contributing traits are considered important and taken as the major determinants in parental selection of high yielding accessions for breeding programmes (Nourin *et al*., 2019) However,

considering the importance and contributions of these characters towards hybridization to improve yield potential of genotypes, the present investigation suggests that with these lower values, this genotype cannot be considered as effective in formulating selection criteria for yield improvement in mungbean germplasm. Therefore, from the breeding point of view, this genotype appears not to be a good candidate for mungbean breeding programmes in Botswana, especially when focusing on yield improvement. In general, the distribution pattern of the evaluated genotypes exhibited by both clustering methods (morphological and SSR) proved the existence of diversity among the 18 evaluated mungbean genotypes.

CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The results of this study showed the existence of genetic variation among the eighteen genotypes available in Botswana's mungbean germplasm collection at both morphological and molecular levels. The level of similarity displayed by most of the analysed morphological traits (quantitative and qualitative) indicated a narrow genetic base among the tested genotypes. Majority of the characters showed greater relevance in these genetic variations and relationships among the evaluated genotypes which included growth habit, leaf colour, raceme position, pod colour, testa texture, days to flowering, peduncle length, grain yield ha^{-1} and harvest index. Even though variation existed, some morphological characters were unable to distinguish some of the tested mungbean genotypes. The observed variation in the evaluated characters gives a scope for manipulation of local mungbean germplasm to start a good hybridization programme in developing more productive varieties. Overall, genotypes E084, E116 and VC1482E outperformed control genotype (Mmelegi) based on various morphological traits and by virtue of their promising performance, they have a great potential and can be used as parents in future mungbean programmes to provide progenies with high genetic variability. The results of this study also displayed significant variation in root-nodule traits and photosynthetic parameters among the studied mungbean genotypes, hence these traits could be manipulated to provide a powerful genetic approach for yield enhancement in mungbean.

6.2 Recommendations

Based on the results of this study, the following recommendations are made;

I. There is a need to explore and exploit more numbers of mungbean germplasm by the Botswana National Plant Genetic Resource Centre (BNPGRC) personnel from different regions across the globe in order to widen the available gene pool to increase genetic diversity of mungbean

II. To complement the morphological and molecular findings, further investigations should be carried out to address the nutritional information to determine the nutritive value of the seeds.

III. A relatively higher number of genotypes should also be considered.

IV. More SSR markers should also be included in the analysis.

V. It would also be worthwhile to evaluate the current studied genotypes in various agro ecological zones of Botswana since this study was conducted in a single environment (one location), with the aim of understanding diversity of mungbean in diverse climatic regions of Botswana.

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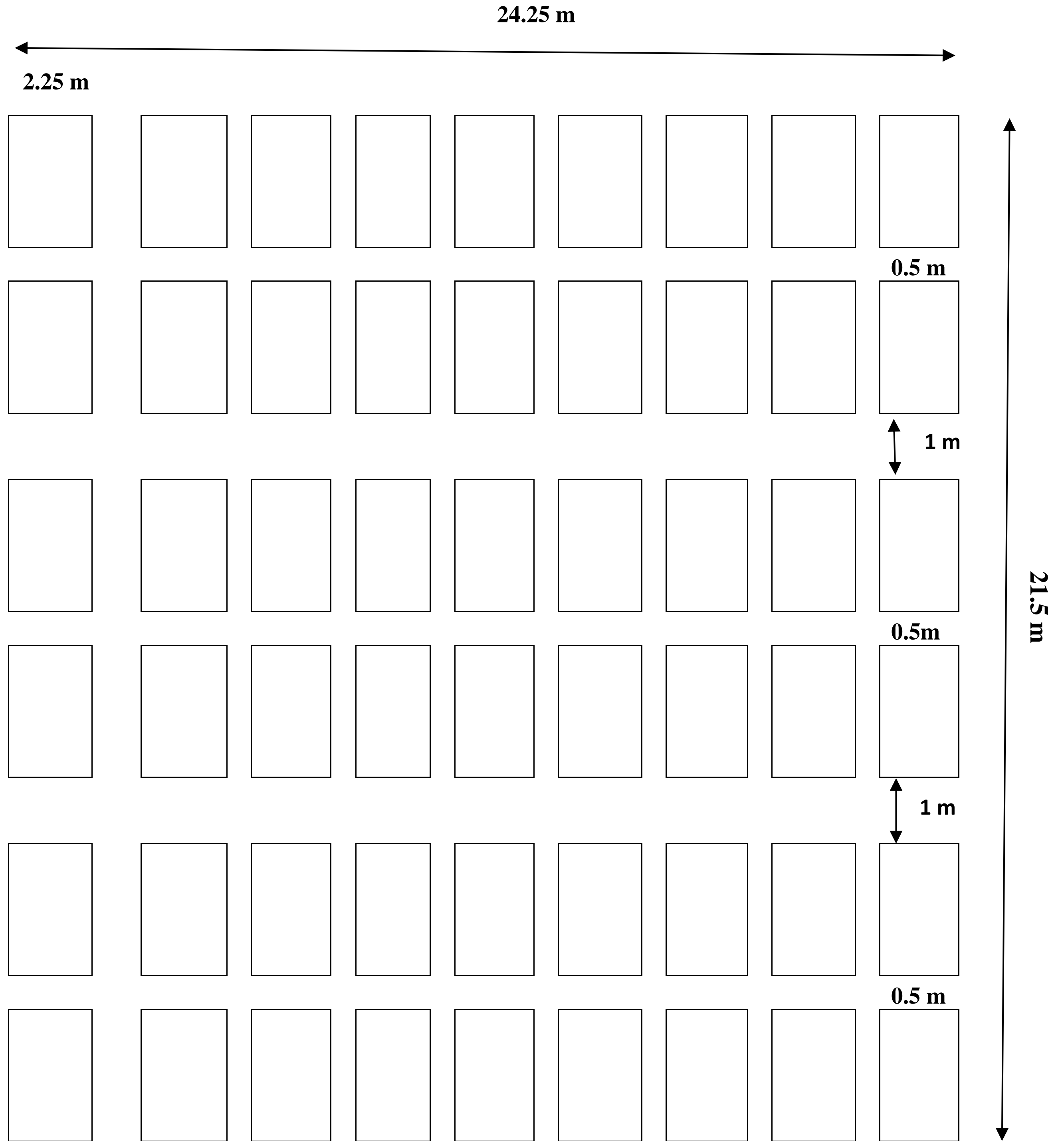
APPENDICES

Appendix 1: Field plot layout showing experimental design



Appendix 2: Growth and development of mungbean genotypes in the greenhouse

Appendix 3: The experimental field of the evaluated mungbean genotypes