GENETIC AND BIOCHEMICAL CHARACTERIZATION OF PEARL MILLET MUTANT LINES USING SIMPLE SEQUENCE REPEAT MARKERS IN NAMIBIA

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ABSTRACT

Pearl millet is a staple food and fodder crop of marginal areas of sub-Saharan Africa, South Asia and the Americas. Although pearl millet is stable food for 70 % of Namibian population, its productivity is very low (low yield, susceptible to diseases/insect and prolonged reproductive cycle) and has a limited genetic diversity. For the purpose of widening gene diversity, developing high yielding and drought resistant pearl millet, the *Kangara* and *Okashana 2* were mutated using Colbalt-60 gamma irradiation source with various dose levels. However, all sixth generation of *Okashana 2* putative mutant derivatives used in this study were all produced using 350 Gray while for *Kangara* mutants were produced using 400 Gray.

A total of 32 genotypes including two parental lines were collected from the three agro-ecological zones (Research Stations). In addition to two parental lines, stratified sampling technique was used to randomly select further 12 samples from 30 putative mutants for *Okashana 2* and *Kangara* that offered higher average grain yields. Such technique used to subsample was applied in order to select putative mutants that shown phenotypically high yielding trait. This study aimed to examine the genetic diversity existing in the Namibian pearl millet putative mutant lines using 19 simple sequence repeats and 23 biochemical markers. Genetic characterization was studied using conventional PCR and separated using gel electrophoresis while biochemical parameters used dumas method (crude protein), fiber extractor (crude fibre, ADF and NDF), fat solvent extractor (fat), ICP spectrophotometer (minerals) and UV/VIS spectrometer (phytate).

A total of 108 putative bands were generated from 11 SSR markers whereas the remaining eight markers were not effective in amplifying any mutant line. Amplified fragment size ranged from 90 to 1150 bp and the 11 SSR loci had a 63.6 % polymorphism. Polymorphic information content (PIC) varied from 0.139 to 0.872, with a mean of 0.379. Cluster analysis using UPGMA revealed a 51.8 % similarity level among the genotypes. The mean Shannon-Wienberg diversity index (H') was 2.589, while the mean Simpson's diversity index (D) was 0.93. Both Simpson's index value and the Shannon-Wiener diversity shown moderate results in both species richness and evenness, while the genetic diversity level of similarity among putative mutants, suggested moderate genetic diversity.

Biochemical parameters of proximate content (moisture, ash, crude protein, fat, crude fibre and carbohydrates) showed no consistency in up/down regulation pattern in comparison to

their parental lines. Only total energy content (however direct correlated with crude protein, crude lipid and carbohydrate) showed a constant trend of down regulation between mutant derivatives and their parental lines. Lines R9P5, L8P5Rep2, L9P3Rep1, L10P18, L5P1Rep2 and L0P3Rep1 are selected for their higher protein content, while L5AP18, L9P3Rep1 and L8P5Rep2 selected for trait such as higher content of WAC and swelling capacity. L5AP18 and R6P1-1 are selected for higher content of iron and zinc minerals. Generally, majority of biochemical markers showed a significant difference ($P < 0.05$) within and among *Kangara* and *Okashana 2* derivatives and as well as in comparison with the parental lines. Crude protein content of the mutant varieties in this study showed significant increment of up to 24 % in comparison with parental lines.

Correlation analysis (r) between SSR and biochemical markers of pearl millet putative mutants ranged between -0.44 - 0.70, but with a low mean correlation analysis ($r = |0.31|$). There is little or no correlation between biochemical markers (proximate composition, mineral nutrient contents, functional and anti-nutrients properties). There was low correlation observed among the proximate composition biochemical markers, while mineral contents, functional and anti-nutrients properties markers showed a moderate correlation. Finally, the study identified valuable and usefulness of the SSR marker system to assess genetic diversity in pearl millet putative mutant germplasms and the number of alleles/bands detected for each SSR marker can be a good indicator of genetic diversity. The result further revealed that putative mutant germplasms are significantly different to their parental lines in terms of genetic diversity and biochemical traits. In most of the parameters, the putative mutants had shown better improvement in comparison to their parental lines. Thus, assessment of genetic diversity and determination of the biochemical activities in pearl millet germplasm should improve the breeding efficiency and allow to understanding the genetic potential of each putative mutant. Genetic characterization of germplasm using molecular and biochemical markers in crop improvement is one of the most sustainable methods to conserve valuable genetic resources and to simultaneously increase agricultural production and food security.

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LIST OF GENERAL ABBREVIATIONS

ANOVA	Analysis Of Variance
AOAC	Association of Analytical Chemists
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
FAO	Food and Agriculture Organization of the United Nations
FAO/IAEA	Food and Agriculture Organization/International Atomic Energy Agency
ICRISAT	International Crop Research Institute for Semi-Arid Tropics
ID	Identification
MAWF	Ministry of Agriculture, Water and Forestry
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SADC	Southern African Development Communities
SSRs	Simple Sequence Repeats
STDV	Standard deviation
TAE	Tris-Acetate ethylenediamine tetraacetic acid
TBE	Tris/Borate/EDTA
UNAM	University of Namibia
UPGMA	Unweighted Paired Group Method Using Arithmetic Average

LIST OF NUMERICAL ABBREVIATIONS

bp	Base pairs
cm	Centimeter
g	Relative centrifugal force
g	Grams
Gy	Gray
hrs	Hours
kg/ha	Kilogram per hectare
mg	Milligrams
mL	Milliliter
min	Minutes
ng	Nanogram
RPM	Revolution per minute
sec	Seconds
°C	Degrees Celsius
µg	Microgram
µL	Microliter
%	Percent

DECLARATION

I, Filemon Nghitilanganye Shindume, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Filemon Nghitilanganye Shindume Date

DEDICATION

This work is dedicated to my special aunty **Rita Mweneni Kaurah** for raising me up with love and taught me to swim upstream with a smile.

Finally, to the darling daughter, **Leonia Nghivelwashisho Iyaloo** who may not understand of how much contribution her smile has guided me to remain positive during challenging time of my study. This is for you with all Dads' love.

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CHAPTER 1
INTRODUCTION

Pearl millet, *[Pennisetum glaucum (L.) R. Br.]* (2n = 2x = 14), is an important crop to most farmers in the tropics and sub-tropics of Africa, South Asia and the Americas (Issoufou, Mahamadou, & Guo-Wei, 2013; Saleh, Zhang, Chen, & Shen, 2013).

This orphan crop was first domesticated in the Sahel zone of West Africa which is known to be one of the world crops main centres of diversity. It has been grown in Africa and on the Indian subcontinent since prehistoric times. Recent archaeological and botanical research has confirmed the presence of domesticated pearl millet in the Lower Tilemsi Valley in north-eastern Mali about 4,500 years ago (Manning, Pelling, Higham, Schwenniger, & Fuller, 2011).

Pear millet belongs to family *Poaceae* that differ either at subfamily, species, or genus levels (Dwivedi et al., 2012; Saleh et al., 2013). There are discrepancies exist concerning classification of family millet, with some references giving the family name *Gramineae*, and others classifying it in the family *Poaceae* (Issoufou et al., 2013). Some argued that millets are classified with maize, sorghum, and Coix (Job's tears) in the grass sub-family *Panicoideae* (Issoufou et al., 2013). The millets family comprises of a number of small-grained, annual cereal grasses that include several distinct species such as pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), little millet (*Panicum sumatrense*), barnyard millet [*Paspalum crusgalli* (Japanese) and *Eleusine colona* (Indian)], kodo millet (*Paspalum scrobiculatum*), tef (*Eragrotis tef*), fonio [*Digitaria exilis* (white fonio) and *D. iburua*

(black fonio)], guinea millet (*Brachiaria deflexa*), and Job's tears (*Coix lacrymajobi*) (Dwivedi et al., 2012; Issoufou et al., 2013; Saleh et al., 2013).

The word millet is derived from the French word "mille" meaning thousand, implying that a handful of millet contains thousands of grains (Taylor & Emmambux, 2008). In Namibian context pearl millet is locally known as "*Mahangu*" with its two main local varieties of *Okashana 2* (SDMV 93032) and *Kangara* (SDMV 92040) and such term will be used interchangeable in this thesis (Ipinge, 1998; Rohrbach, Lechner, Ipinge, & Monyo, 1999).

Pearl millet growing cycle varies from 48 to 120 days, characterized by a panicle that takes many forms with a female flowering earlier than a male and pollinated by wind (Lemgharbi et al., 2016). It is highly outcrossing crop irrespective of more than 85 % of its flowers are hermaphrodite due to its protogyny nature of its allogamous flower (Dwivedi et al., 2012; Saleh et al., 2013). Pearl millets are robust with extensive root system and upright bunch grass that tillers from the base. It often grows to a height of 3 meters, although the most productive hybrids and improved open-pollinated varieties are short stature (Dwivedi et al., 2012). Cultivated pearl millet displays tremendous phenotypic variability for traits such as flowering time, panicle length, grain and stover characteristics, tolerance to drought, pests, and diseases, as well as nutritional value (Stich et al., 2010). Pearl millet is diploid sexually propagated plant with 22 chromosomes and a large genome size of 2450 Mega base pairs (Varshney, Ribaut, Buckler, Tuberosa, Rafalski, & Langridge, 2012).

Pearl millet is the sixth most important cereal crop after rice, wheat, maize, barley and sorghum, globally and it is a staple crop for over 70 % of the Namibian population (Dwivedi et al., 2012; McBenedict, Chimwamurombe, Kwembeya, & Maggs-Kölling,

2016). Due to its drought and high temperature tolerance, pearl millet is widely cultivated under rain-fed but also under irrigation in hot and drought-prone regions (Sehgal et al., 2012). It is usually grown for its nutritious grain for human consumption and poultry feed as well as fodder for livestock and as construction materials (Sehgal et al., 2012).

Out of 255.1 million tonnes global cereal production from 2004 to 2008, pearl millet constituted 12.7 % which is around 32.3 million tonnes (Dwivedi et al. 2012). Regional performance indicated that pearl millet was cultivated on about 2 million hectares in the Eastern and Southern Africa and that contributed to 50 % of pearl millet's share of global millet productions (http://exploreit.icrisat.org/page/pearl_millet accessed on 26 May 2017). In Namibia, *Okashana* 1 and *Okashana* 2 pearl millet varieties have been adopted on a large scale covering more than 50 % of the total pearl millet area prior to 2008. In Namibia, *Mahangu* is the leading crop in cultivated area followed by white maize and wheat (Nakusera, Kadhikwa, & Mushendami, 2008). In 2004, about 96, 370 tons of *Mahangu* were produced compared with the 55,597 tons of maize in the same year (Nakusera et al., 2008).

Pearl millet production has declined recently due to abiotic (drought, temperature, salinity and low nutrient supply) and biotic (mainly diseases) factors (Bado et al., 2015). To enhance crop production and productivity, the Government of Namibia in collaboration with the International Atomic Energy Agency (IAEA) initiated a mutation breeding project during 2007 (Horn & Shimelis, 2013). This project is being coordinated by the Namibian Ministry of Agriculture, Water and Forestry (MAWF). Through this initiative seeds of the two traditional pearl millet varieties (*Okashana 2 and Kangara*) were gamma irradiated with varied doses. Selection of genotypes started from M2 and the putative mutants used in this study were selected from M_6 generation.

As part of the initiative, the present study was undertaken to characterize the newly developed putative mutants using molecular and biochemical markers. Characterization of genetic resource collections has been greatly facilitated by the availability of a number of biochemical and molecular marker systems, but no single method is adequate for assessing genetic variations (El-Nahas, El-Shazly, Ahmed, & Omran, 2011). Application of different characterization methods increase the efficiency of detecting sample variations as well as improve quality of the information content. Genetic and biochemical characterization techniques of mutant crop varieties have been successfully applied in some countries such as Kenya, Niger, China and India (Omondi et al., 2012; Shu, 2009).

Genotyping is the term that is used to describe the process of using laboratory methods to determine genetic variability at DNA level and detect gene/allele variants that an individual organism carries usually at one particular gene or locus in the genome (Shu, Forster, & Nakagawa, 2012). Genotyping/fingerprinting of putative mutant genotypes with promising agronomic traits could contribute to further improve the crop. Biochemical characterization, on the other hand, is a technique used to detect diversity at functional gene level. It is against this background that this thesis will focus on genotypic and biochemical characterization of the selected pearl millet putative mutant germplasms.

1.1 Statement of the problem

Since Namibian independence only two varieties of pearl millet, one sorghum and three cowpea varieties were introduced to Namibia from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and the International Institute of Tropical Agriculture (IITA) germplasm selection (Horn, Shimelis, & Laing, 2015). Despite the release of these varieties, there are limited studies using the application of molecular and

biochemical markers on these released crop varieties with promising agronomic traits especially on pearl millet. Therefore, understanding the genetic makeup and biochemical characteristics of the crop will be useful to breeders and nutritionists. This study is part of a large ongoing project on "Developing high yielding and drought resistant pearl millet *[Pennisetum glaucum (L.) R. Br.]*, sorghum *[Sorghum Bicilor (L) Moench]*, bambara groundnut (*Vigna subterranea*) and cowpea [*Vigna unguiculate (L) Walp*] varieties in Namibia."

This project is funded by joint Food and Agriculture Organization of the United Nations/International Atomic Energy Agency (FAO/IAEA) and the Ministry of Agriculture, Water and Forestry (MAWF) of Namibia (Horn, Ghebrehiwot, & Shimelis, 2016).

1.2 Objectives of the research

- To evaluate the genetic diversity of selected putative mutant pearl millet lines with improved agronomic traits using SSR markers.
- To examine the biochemical markers (proximate content, nutritionally valuable minerals, anti-nutrients and functional properties of the seed flour) of selected putative mutant pearl millet lines with improved agronomic traits to be used in dietary formulation.

1.3 Significance of the study

Knowledge about the extent of genetic variability caused by induced mutation and biochemical variation in pearl millet putative mutant are an important prerequisite for efficient crop improvement. Also, application of SSR markers and biochemical essay may well be used to identify *P. glaucum* genotypes which are useful in resource management for germplasm conservation. Furthermore, acquired data could provide diagnostic molecular breeding with additional information thus further reduce deficit experienced in screening of crop varieties within Namibian breeding sector.

CHAPTER 2
LITERATURE REVIEW

2.1 Background of pear millet parental lines and their putative mutant derivatives

Main mandate of Namibia's pearl millet breeding program is to provide farmers with earlier-maturing varieties that offer relatively high grain yields in drought conditions. These varieties allow farmers, who planted at the first rains, to obtain a grain harvest within 30 to 50 days earlier than traditional varieties (Rohrbach et al., 1999). Therefore, the new varieties of *Kangara* and *Okashanas* had been introduced in early 1990s. Those varieties allows multiple planting, so that farmers can distribute their labour more evenly over the cropping season and thus improve the timelines of planting, weeding, and harvesting.

Okashana 2 (SDMV 93032) is an early-maturing, high-yielding variety with large and hard vitreous grain and strong stalks. It is developed by multiple institutions to overcome the stalk lodging and germination on the panicle, if exposed to sufficient rainfall associated with its parental line (*Okashana 1)* (Rohrbach et al., 1999). The parent material, *Okashana 1*, is originated in Togo and improved by ICRISAT-India, and further improvements have been done by Sorghum and Millet Improvement Program (SMIP) in Zimbabwe and Namibia. However, *Kangara* (SDMV 92040) pearl millet variety was developed by Southern African Development Community/ International Crops Research Institute for the Semi-Arid Tropics (SADC/ICRISAT) and SMIP at Matopos Research Station in Zimbabwe (Ipinge, 1998; Rohrbach et al., 1999). According to Rohrbach et al. (1999) this variety was developed by ICRISAT-Patancheru through inter hybridization of different germplasm collected from Southern Africa and Togo.

This variety is characterized by its large bold creamy white grains and early maturity within 50 to 55 days (Ipinge, 1998; Rohrbach et al., 1999).

For the purpose of developing high yielding and drought resistant pearl millet, the dry, healthy and quiescent seeds of *Kangara* and *Okashana 2* were prepared for irradiation after collected from National Plant Genetic Resource Centre of Namibia (Gene Bank of Namibia). Prior to gamma ray treatment, preliminary germination and viability tests were conducted and provided 100 % germination rate (Horn & Shimelis, 2013). The treatment was conducted at the International Atomic Energy Agency (IAEA), Agriculture and Biotechnology Laboratory, A-2444 Seibersdorf, Austria. The seeds were packed in separate seed envelopes and placed in desiccators for three days to attain the desired moisture level of 8 % (Horn & Shimelis, 2013). Then, thirty seeds per genotype were gamma irradiated in three replications using a Colbalt-60 gamma source, Gammacell, Model 220.

Various doses level were used to establish the optimum mutation frequency with least possible and unintended damage (Mba, Afza, Bado, & Jain, 2010). However, all sixth generation of *Okashana 2* putative mutant used in this study were all produced using 350 Gray (Gy) dose level while the Kangara putative mutants were produced using 400 Gy.

2.2 Pearl millet utilization

In many African and Asian countries, millets serve as a primary food commodity and various traditional foods and beverages, such as bread (fermented or unfermented), porridges, and snack foods, are made specifically among the non-affluent segments in their respective societies (Issoufou et al., 2013; Saleh et al., 2013). However, in the North

America and Europe, millets are not the primary nutritional source but they serve as major ingredients in multigrain and gluten-free cereal products (Saleh et al., 2013). Over 95 % of pearl millet production was used as food and the remaining 5 % are being divided between animals and poultry feed (Deepak, Niranjan-Raj, Mithöfer, & Shetty, 2012).

2.3 Importance of pearl millet improvements

The food demand to feed the ever growing populations of the world (projected to exceed 9 billion by the year 2050) have so far outstripped the 37 % linear rate of increases in food production and it is estimated that 70 % more food must be produced over the next four decades in order to adequately meet such demands (Mba, Guimaraes, & Ghosh, 2012). The challenge of producing enough food for a rapidly growing population under extreme and unpredictable weather conditions is further exacerbated by dwindling agricultural land and water resources. It is further aggravated by the demand for bio-energy crops while minimizing the costs of carbon production (Mba et al., 2012; Parry et al., 2009). One of the main solutions is intensifying agricultural products through introduction of improved crop cultivars that are more resilient to climate changes with improved agronomic traits such as high yield, well-adaptation, high input use-efficient (Bado et al., 2015).

Globally, pearl millet is the sixth most consumed cereal crop in the world (Dwivedi et al., 2012), and it is a staple crop for over 70 % of the Namibian population. However, pearl millet often suffers from undesirable production traits such as susceptibility to diseases/pests, low yield, and prolonged reproductive cycle (McBenedict et al., 2016). These need to be improved to avert threat to the nation's food security. McBenedict et al. (2016) also reported that the low level of genetic diversity among Namibian collections of pearl millet accessions hinders the improvement of the crop. Furthermore, Hu et al. (2015)

reported that accessions from southern Africa and Asia suffered from narrow genetic base. As a result efforts must be made to widen the available gene pool in order to increase the genetic diversity of pearl millet in the country.

Most common breeding technique is hybridization and recurrent selection to recombine and integrate favourable genes into elite genotypes using the available germplasm (Oladosu et al., 2016). Due to the inherent low genetic variability existing within a species and among cultivated or improved cultivars, the genetic improvement of the crop is so slow. Therefore, conventional breeding often fails to provide desirable results and additional approaches, such as mutation breeding and genetic engineering need to be employed to widen the genetic base in order to address food security and sustainability in agriculture (Oladosu et al., 2016).

Genetic engineering is one of the most efficient methods to introduce specific, targeted and improved agronomic traits into crop species. Because of the high initial investment, social controversial issues and the many regulations, including intellectual property right, the application of this method is only restricted to private companies. Another simple and cost effective approach is mutagenesis, an important tool in crop improvement and free of the regulatory restrictions and licensing costs (Kenzhebayeva et al., 2014; Parry et al., 2009). Therefore, induced genetic diversity is a proven strategy in the improvement of all major food crops. The application of mutagenesis to create novel variation is particularly valuable in crop species with narrow genetic base (Parry et al., 2009). The prime strategy in mutation-based breeding has been to improve one or two major traits of well-adapted plant varieties by altering one or few genes (Mba et al., 2012; Pathirana, 2011). These include characters such as plant height, maturity, seed shattering, and disease resistance,

which contribute to increased yield and quality traits (modified oil profile and content, malting quality, and size and quality of starch granules) in various crops.

As a result, mutation breeding has contributed immensely to the development of genetically improved crop varieties (Suprasanna, Mirajkar, & Bhagwat, 2015). However, unlike hybridization and selection, mutation breeding has the advantage of improving a defect in an otherwise elite cultivar, without losing its agronomic and quality characteristics (Pathirana, 2011). It is obvious that the development and selection of varieties meeting specific local food and industrial requirements from available or created genetic diversity is important for food security in Namibia and in the world in general (Omondi et al., 2012).

During 1970 - 2006, only 15 % productivity increase (from 800 kg/ha to 920 kg/ha) in the southern African region of pearl millet was observed (http://exploreit.icrisat.org/page/pearl_millet retrieved on 26 May 2017). In Namibia, pear millet yield (127 kg/ha) is by far lower than the global and India's average (largest producer of pearl millet in the world) yield of 1310 kg/ha and 1044 kg/ha, respectively (Tara Satyavathi et al., 2013). In contrast, the human population of Southern African region has increased by more than 94 % (from 127.42 million to 247.49 million) during 1980 - 2006 periods (http://www.sadc.int/information-services/sadc-statistics/sadc-statiyearbook accessed on 26 March 2017). According to the Namibian Agronomic Board, in the year 2014/15 planting season, the actual total production of pearl millet in *Mahangu* was 28, 584 tons from a total area of 225, 831 hectares planted (http://www.nab.com.na/controlled-crops/grain/mahangu accessed on 26 October 2016). Unexpectedly, the yield figure is way too low and one may speculate only that such figure maybe highly influenced by the number of 'subsistence' farmers that planted pearl millet

that seems unable to adequately produce the crop due to different challenges during cultivation. These include the utilization of traditional farming practices, their inability to access improved varieties and capitals. Might be further mitigated by drought as *Mahangu* is cultivated in a drought-prone area and farmers mainly cultivate pearl millet using rain-fed cropping system.

2.4 Artificially induced mutation and the breeding impact

Plant breeding began as early as 10,000 BC during the Neolithic revolution, when tribes of hunter-gatherers started their shift towards a sedentary and agrarian society (Sikora, Chawade, Larsson, Olsson, & Olsson, 2011). Therefore, artificially induced mutation breeding that has been used since the 1930s is simply a new device in the breeder's toolkit (Ahloowalia, Maluszynski, & Nichterlein, 2004).

Mutation breeding is a non-transgenic technique that has been used as an efficient tool for crop improvement that can meet the increasing demand in crop production in various countries (Pathirana, 2011). This technique make use of mutagenic agents such as alkylating agents, gamma rays, X rays, UV light, fast and thermal neutrons, beta and alpha particles. However, artificially induced mutation breeding using gamma radiation has been the most frequently used method for directly developing mutant varieties (Ahloowalia et al., 2004). Many researches indicated that physical mutagens, mostly ionizing radiations, have been widely used for inducing genetic aberrations in the past 80 years. It has been reported that 70 % of mutant varieties were developed using physical mutagenesis (Oladosu et al., 2016).

Mutants are created by exposing genetic materials to different dose level of gamma irradiation. Gamma and X-rays are the most commonly used physical mutagens (Shu et al., 2012). Gamma rays are emitted in the process of the decay of the radioisotopes cobalt-60 (^{60}Co), cesium-137 (^{137}Cs) and to a less extent, plutonium-239 (^{239}Pu) (Mba, 2013). Gamma sources containing one of these radioisotopes are typically installed as gammacell irradiators and are used mostly for acute irradiation. Gamma rays are a highly energetic rays that are deposited in cellular structures of seeds through discrete ionization events that are essentially randomly distributed in space (Manova & Gruszka, 2015).

Gamma radiation causes a wide spectrum of chemically different types of lesions in DNA. Such lesions of the so-called locally multiply damaged sites (LMDS) may consist of single-strand breaks (SSB) on opposite strands or if located close to each other it may give rise to double-strand breaks (DSB) (Bray & West, 2005; Mba, 2013; Olasupo, Ilori, Forster, & Bado, 2016; Pfeiffer, Goedecke, & Obe, 2000).

Other LMDS containing chemically modified base and sugar moieties have the potential to cause DSB following strand cleavage by cellular base damage-specific enzymes (Manova & Gruszka, 2015). Thus, DSB induced by gamma irradiation may arise as a direct consequence of one or more ionizing events or indirectly as a consequence of repair processes that eliminate closely spaced base or sugar damage on opposite strands (Dexheimer, 2013; Gill, Anjum, Gill, Jha, & Tuteja, 2015; Pfeiffer et al., 2000).

Due to the presence of complex chemical alterations in the DNA, DSB generated by gamma rays are particularly challenging to repair and this increases the rate of potential mutations (Pfeiffer et al., 2000). Radiation can increase the natural mutation rate by 1000 to 1 million fold, making the generation of genetic variation very effective (Shu et al., 2012). Therefore, physical agents of gamma radiation using Colbalt-60 gamma source

(dose range from 350 Gy to 400 Gy) was used to produce the pearl millet putative mutant lines used in this study.

Around 3246 mutant varieties from 214 different plant species in more than 60 countries are officially released (https://mvd.iaea.org accessed on 18 Feb. 2017). Of which over 1,000 mutant varieties were created from major staple crops, cultivated on tens of millions of hectares, enhance income, improve human nutrition and contribute to environmentally sustainable food security in the world (Mba et al., 2012; Oladosu et al., 2016; Parry et al., 2009).

However, only five mutant pearl millet varieties official released in the world since 1974 and all have fungal diseases resistance traits (https://mvd.iaea.org accessed on 18 Feb. 2017). Therefore, several achievements in crop improvement through mutation breeding have resulted in improved varieties that are directly used for commercial cultivation and new genetic stocks with improved characters (Oladosu et al., 2016).

Furthermore, induced mutation breeding techniques helped to develop many agronomical important traits of shorter growing period, higher yield, improved quality, suitable for rotation, post-harvest degradation, increased tolerance/resistance to abiotic and biotic stresses and novel end-user characters use in crops of interest (Kenzhebayeva et al., 2014). It might be fair to conclude that mutation techniques in plant breeding programmes throughout the world has generated thousands of novel crop varieties in hundreds of crop species, and billions of dollars in additional revenue (Mba et al., 2010).

2.5 DNA damages and repair mechanisms in mutation

DNA is the most stable hereditary material in all living organisms. However, it is an evolutionary dymanic molecule that is constantly challenged by endogenous and exogenous DNA-damaging factors (Gill et al., 2015; Manova & Gruszka, 2015). Sometimes because of environmental stress factors (physical and chemical mutagenic agents) and DNA replication errors, it is subject to assault that leads to mutation (Clancy, 2008; Dexheimer, 2013; Pray, 2008). Naturally, all most all living organisms have highly coordinated mechanism to repair any changes that is created in their genomes. Cells initiate a highly coordinated cascade of events, collectively known as the DNA damage response (DDR), in which the system detects the DNA damage, prepare the cell regulatory system by sending signals and mediates its repair (Dexheimer, 2013).

In an organism, the spontaneous mutation rate is determined by the rate at which DNA polymerase adds incorrect nucleotides during DNA replication and miss detected by the proofreading enzyme (Clancy, 2008). All the errors created during DNA replication is corrected by a proofreading enzyme, it normally recognizes the errors and corrects immediately. However, some mutations survive this process and as a result new variants (mutants) can be created (Pray, 2008). The diagram illustrates common DNA damaging agents, examples of DNA lesions caused by these agents, and the relevant DNA repair mechanism responsible for their removal (Figure 1).

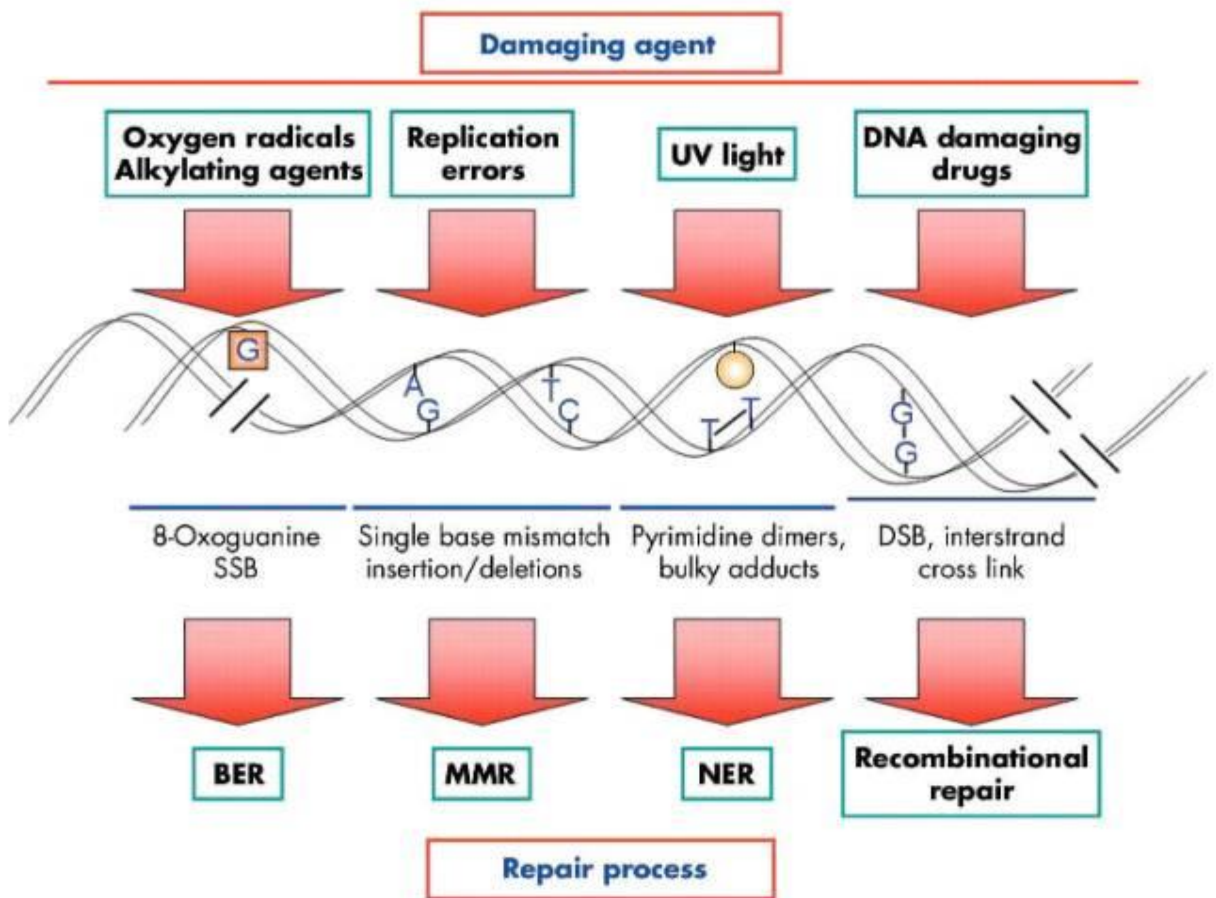


Figure 1 Types of DNA damages and repair mechanisms (Dexheimer, 2013).

DNA damage can be classified into covalent modification of DNA bases (deamination i.e. cytosine converted to a uracil), mismatches due to proofreading failure during DNA replication, breaks in the backbone (both single- and double-strand break) and cross links covalent linkages (Dexheimer, 2013). Generally, many forms of DNA damage affect only one of the two strands of the duplex. Therefore, highly accurate repair of DNA lesions is facilitated using the sequence information provided by the complementary strand (Bray & West, 2005). For organism’s survival, DNA repair is closely tied to regulation of the cell cycle during which the checkpoint mechanisms ensure that a cell's DNA is intact before

permitting DNA replication and cell division to occur (Branzei & Foiani, 2008). A variety of different DNA repair pathways have been reported that include direct reversal, base excision repair (BER), nucleotide excision repair (NER), photo-reactivation, bypass, double-strand break repair, and mismatch repair (MMR) pathway (Tuteja, Singh, Misra, Bhalla, & Tuteja, 2001).

The repair mechanism of base excision repair (BER) is the predominant mechanism that handles the spontaneous DNA damage (oxidized, alkylated, or hydrolysed) caused by free radicals and other reactive species generated by metabolism ((Dexheimer, 2013; Clancy, 2008). All such changes result in abnormal bases that must be removed and replaced using lesion-specific DNA glycosylases. Such enzymes remove damaged bases by literally cutting them out of the DNA strand and then fill the resulting gap by a specialized repair polymerase before sealing by ligase.

Another method employed by the cell is a nucleotide excision repair (NER) which is a highly versatile repair pathway that can recognize and remove a wide variety of bulky, helix-distorting lesions from DNA (Dexheimer, 2013; Gill et al., 2015; Manova & Gruszka, 2015). While mechanistically similar to BER, the NER pathway is more complex, that involves DNA damage recognition, local opening of the DNA helix around the lesion, excision of a short single-strand segment of DNA spanning the lesion, and sequential repair through synthesis and strand ligation (Bray & West, 2005; Gill et al., 2015; Manova & Gruszka, 2015; Singh, Sujit, Swarup, & Dibyendu, 2010).

The mismatch repair (MMR) system plays an essential role in post-replication repair of mis-incorporated bases that have escaped the proofreading activity of replication polymerases. In addition to mismatched bases, MMR proteins also correct insertion/deletion loops that result from polymerase slippage during replication of

repetitive DNA sequences (Dexheimer, 2013; Singh et al., 2010). This pathway is highly important because MMR deficient cells are said to display a mutator phenotype, which is characterized by invariably microsatellite instability and an elevated mutation frequency (Bray & West, 2005; Manova & Gruszka, 2015). This DNA repair mechanism is not helpful in creating crop variation/mutation as it minimizes point mutation. Therefore, if proofreading fixes about 99 % of the errors, mismatch repair even further reduces the final error rate after replication (Pray, 2008).

One form of DNA damage is double-strand breaks (DSB), which is among the most biologically hazardous types of DNA damage caused by ionizing radiation (Clancy, 2008; Dexheimer, 2013). This form of DNA damage is of interest to breeders since the mutant lines used in this study were created using this technique. In addition to interfering with replication, it is highly deleterious that can lead to genes or chromosomal rearrangements in which pieces of one chromosome become attached to another chromosome (Dexheimer, 2013). DNA DSBs elicit a profound cellular response that involves activation of cell cycle checkpoints to facilitate DNA repair, or the activation of apoptosis in situations where cell loss can be tolerated via cell replacement (Phillips & McKinnon, 2007).

When DSB is detected, two different repair mechanisms, non-homologous end joining (NHEJ) or homologous recombination repair (HRR), may be initiated to repair the lesion. These pathways are largely distinct from one another but function in complementary ways to effect DSB repair (Jackson, 2002). These pathways are responsible for balancing genome stability against the generation of genetic diversity (Bray & West, 2005).

In yeast, three DNA repair models have been recognized that are pertaining to HRR, i.e. DNA double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) and single-strand annealing (SSA) model (Gill et al., 2015; Manova & Gruszka, 2015).

During HRR, the homologous chromosome is used as a template for repair and largely an error-free mechanism as it utilizes the genetic information contained in the undamaged sister chromatid as a template. HRR can also be error prone if not restricted to the identical locus on a sister chromatid (Manova & Gruszka, 2015).

DSBR model of HRR is initiated by a DSB and mediates Holliday junction formation, crossing over between aligned homologous chromosomes rather than sister chromatids (Bray & West, 2005; Lieber, 2010; Singh et al., 2010). Initially, the ends of the duplex at the DSB are extensively resected to produce long 3′ tails that invade the homologous chromosome (Gill et al., 2015; Jackson, 2002; Pfeiffer et al., 2000). DNA synthesis is primed from the invading strand using the homologous chromosome as a template. Ligation of the invading strand with the other side of the DSB results in the formation of a joint molecule containing two Holliday junctions which can be resolved with or without crossing over (Gill et al., 2015).

While SDSA model plays important roles in DSB repair in somatic cells (Pfeiffer et al., 2000). This pathway can use a sister chromatid, homologous chromosome or ectopic region of homology in the genome as a recombination substrate (Bray & West, 2005; Lieber, 2010). SDSA behave as DSBR model, contrasting mainly that the newly synthesised DNA then re-anneals with the other side of the DSB, repairing the break and avoiding the formation of the joint molecule. It reduces the likelihood of Holliday junction formation and crossing over, which would be highly mutagenic if this occurred between ectopic regions of homology.

Single-strand annealing (SSA) may occur between tandemly repeated sequences, where homologous regions exposed during resection anneal by single-strand annealing and the

intervening sequence is deleted (Gill et al., 2015; Lieber, 2010). This pathway may be of particular importance on regions of the genome containing repeated gene arrays.

Therefore, HRR pathways in plants can employ one of three DSBs repair mechanism. However, recombination frequencies vary greatly, depending on the origin of the donor and recipient sequences. For instance, situations where homologous sequences abundant, if a break occurs between tandem repeats, a third of DSBs are repaired by SSA and around 7 % are repaired by SDSA (Lieber, 2010).

NHEJ is the major pathway for DSB repair in plant. It does not require homologous sequences; rather, it acts to ligate the two end breaks and often results in deletions or mistakes and thus mutations (Gill et al., 2015). Initial recognition of the DSB is mediated by a heterodimer of KU70/KU80 0 forming a ring structure, which binds exposed both DNA ends and prevents their degradation (Bray & West, 2005; Pfeiffer et al., 2000). The DNA ends may then be processed (possibly by the MRN complex) to make them suitable substrates for DNA ligase (Singh et al., 2010). Processing can involve the alignment of DNA ends at microhomologies of one or more bases and trimming of overhanging DNA 'flaps' (Manova & Gruszka, 2015).

Ligation is then catalysed by a complex of DNA ligase IV and XRCC4 by using overhanging pieces of DNA adjacent to the break to join and fill in the ends (Gill et al., 2015; Jackson, 2002; Manova & Gruszka, 2015; Pfeiffer et al., 2000). Furthermore, other pathways of illegitimate recombination are likely to be present in plant cells (Singh et al., 2010). Although NHR is obviously error prone and degraded or even inappropriate ends may be re-joined, this repair system appears to be crucial in radio-induced DSB repair in plants (Dexheimer, 2013; Gill et al., 2015). Regarding the several pathways described

only SSA and NHEJ that does not depend on the presence of sister chromatids and therefore have a high mutagenic potential (Pfeiffer et al., 2000).

2.6 Screening of mutagenic populations

Mutant screening involves the evaluation of a large number of mutant plants to identify the rare mutant individuals that meet the selection criterion such as improved resistance to disease or a better quality (Shu, 2009). There are two approaches, the forward and reverse techniques. The forward genetic approach enables the identification of improved or novel phenotypes that can be exploited in conventional breeding programmes. On the other hand, reverse genetic approach allows the detection of induced point mutations in individuals of the mutagenized populations, and can address the major challenge of linking sequence information to the biological function of genes and can also identify novel variation for plant breeding (Parry et al., 2009).

The procedures of screening of putative mutant lines ranges from simple physiological parameters for visual screening such as plant stature, flowering time, abiotic and biotic resistance, to more elaborated investigations of seed or fruit quality including genetic characteristics (Bado et al., 2015; Mba et al., 2012; Suprasanna et al., 2015). Elaborated investigations/identification of the mutant lines is required for variety registration, preventing misappropriate crop varieties, protection of plant breeders and farmer's rights (Tara Satyavathi et al., 2013). Therefore, the precise evaluation of the genetic diversity of germplasm provides a guide line for crop genetics and breeding.

The screening techniques can be classified as morphological, cytological, biochemical and molecular approaches. The morphological approach is the simplest and the cheapest techniques that generally represent genetic polymorphisms which are easily detectable

using visual observations and measurements (Xu, 2010). This technique usually used to detect morphological variants among genotypes and used in the construction of linkage maps (Jiang, 2013). Morphological markers can be used to cluster genotypes in different groups and in some cases about 78 % similarity was observed between clusters constructed using morphological and SSR markers, and therefore this can be used as a useful tool to study gene diversity in pearl millet (McBenedict et al. 2016).

The second approach is the use of cytological markers that are the structural variation observed in chromosomes using karyotype. These includes variation in banding patterns displayed in colour, width, order and position that reveal the difference in distributions of euchromatin and heterochromatin (Jiang, 2013). These chromosome landmarks are also widely used in physical and linkage mapping. It is reported that direct use of cytological markers has been very limited in genetic mapping and plant breeding (Jiang, 2013; Xu, 2010). A recent study by Lemgharbi et al. (2016) used cytological markers to successful group pearl millet genotypes based on their panicle characters and seed nutritional quality.

Thirdly, biochemical/protein markers are alternative forms or structural variants of an enzyme that have different molecular weights and electrophoretic mobility but have the same catalytic activity or function (Jiang, 2013). They are used in seed purity test as there are only a small number of isozymes in most crop species and therefore, the use of enzyme markers is limited (Jiang, 2013). The isozymatic markers were successful applied in testing for stability of the patterns in pearl millet (Neto, Pinho, Carvalho, Pereira, & Neto, 2013) as well as to characterize pearl millet into four clusters based on less than 50 % Jaccard's similarity coefficients (Manivannan, Chhabra, & Nimbal, 2013). Other authors reported that protein electrophoresis was used to characterize downy mildew resistant in pearl millet genotypes based on the banding pattern and the similarity index (Sapre, Singh,

Saripalli, Patil, & Talati, 2014). Esterases were also found to be suitable for testing hybrid purity and they are reported to be a possible descriptor for testing distinctness, uniformity and stability (DUS) of new pearl millet genotype (Kumar, Sherry, Varier, & Sharma, 2004)

Finally, DNA markers are marker systems used for evaluating multiple traits simultaneously, at any developmental stages, using DNA from all kinds of plant organ or tissue, and independent of the influence of environmental conditions (Shu et al., 2012). Several marker systems have been developed: Restriction Fragment Length Polymorphism (RFLP), non-PCR based DNA markers and mainly used for genome mapping and comparative genomics and synteny mapping (Xu, 2010). RFLP markers are co-dominant, locus-specific and highly reproducible markers with no prior sequence information, easily applicable and no special equipment required (Jonah et al., 2011). However, its application is limited because large amount of high-quality DNA and blotting/hybridization steps required. It is also very difficult to automate and interpret complex RFLP allelic data in the absence of sequence information together with its inherent low throughput genotyping capacity make its application unattractive. In the last decades, the high radioactive autography involving in genotyping and physical maintenance of RFLP probes and impracticality of sharing RFLP data among laboratories, makes its application even more difficult among geneticists and plant breeders (Jiang, 2013).

The other marker systems is Randomly Amplified Polymorphic DNA (RAPD) marker, a PCR based DNA markers that detects sequence polymorphism in the total genomic DNA of an individual using a single and a short (mostly 10 bp long) random primer. The primers are non-species specific and can be universal (Jiang, 2013; Jonah et al., 2011). Other advantages of RAPD are the use of relatively small amount of DNA and its higher

levels of polymorphism. Unlike RFLP, the procedure of RAPD markers can be automated and this marker systems do not need DNA probes or radioactive assays or blotting techniques (Jiang, 2013; Jonah et al., 2011; Suprasanna et al., 2015).

RAPD products can be cloned, sequenced and then converted into types of PCR-based markers, such as Sequence Characterized Amplified Region (SCAR), Single Nucleotide Polymorphism (SNP) (Jiang, 2013). Moreover, it is extremely sensitive technique and the use of fluorescent dye primers for automated fragment analysis and software packages to analyse the biallelic data makes it well suitable for high throughput analysis. Other strength includes high level of polymorphism, require small amount of genomic DNA, no sequence information is required, set of primers can be used for different species, very high multiplex ratio and its relatively reproducibility across laboratories (Jiang, 2013; Jonah et al., 2011; Xu, 2010). However, RAPD is a dominant marker with limitations of low reproducibility/repeatability (Jiang, 2013; Jonah et al., 2011; Suprasanna et al., 2015). Sapre et al. (2014) managed to determine the genetic diversity among downy mildew resistant pearl millet genotypes using RAPD markers.

Amplified Fragment Length Polymorphism (AFLP) is a PCR based DNA markers applicable in biodiversity studies, analysis of germplasm collections, genotyping of individuals, identification of closely linked DNA markers, construction of linkage maps, construction of physical maps, gene flow studies, plant variety registration, gene mapping and transcript profiling (Jiang, 2013). It shares some characteristic with both RFLP and RAPD analysis. It combines the specifically of restriction fragments analyses with PCR amplification of total double-digest genomic DNA under high stringency conditions. As a result, is also called selective restriction fragment amplification (SRFA) (Jiang, 2013; Jonah et al., 2011).

Drawback of AFLP include polymorphic information content (PIC) for biallelic markers is limited to maximum of 0.5, require high quality DNA for complete restriction enzyme digestion, complicated marker development and not cost-efficient, especially for locus-specific markers (Suprasanna et al., 2015). Many studies have reported that the use of AFLP markers to detect gene diversity in Palmer Amaranth (Chandi et al., 2013), sesame (Ali, Yasumoto, & Katsuta, 2007) and pearl millet (Guthridge et al., 2001; Singru, Sivaramakrishnan, Thakur, Gupta, & Ranjekar, 2003), as well as characterise the distribution of AFLP fragments across the genome and particularly in relation to gene locations (Caballero et al., 2013).

Simple Sequence Repeats (SSRs) also called microsatellites, Short Tandem Repeats (STRs) or Sequence Tagged Microsatellite Sites (STMS), are PCR-based markers that are ideal for genetic diversity analysis and population studies (Jiang, 2013; Jonah et al., 2011). It consists of a variable number of tandem repeats of a simple motif sequences, typically a mono-, di-, tri- or tetra-nucleotide repeats. These repeat motifs are flanked by conserved nucleotide sequences from which primers can be designed to amplify the DNA section containing the SSR (Jiang, 2013; Xu, 2010; Yadav, Mitchell, Zamora, Fulton, & Kresovich, 2007). Advantages of SSRs includes its abundance (average frequency about one every 6.04 kb in plant genomic), reproducible, highly polymorphic, co-dominant markers that distinguish between homozygous and heterozygous individuals, and require a small quantity of DNA for PCR analysis (Cardle et al., 2000; Jiang, 2013; Jonah et al., 2011; Shehata, Al-Ghethar, & Al-Homaidan, 2009; Xu, 2010; Yadav et al., 2007).

SSR markers can be multiplexed, automated and have high throughput genotyping (Jiang, 2013; Xu, 2010). However, it requires prior knowledge of the genomic sequence to design specific primers, marker development process is labour intensive and high establishment

costs for automated detections of SSR alleles and thus, SSR markers were limited primarily to economically important species (Jiang, 2013; Shehata et al., 2009; Xu, 2010).

Some other microsatellite markers that have been used on the same principle are developed and they include the Randomly Amplified Microsatellite Polymorphism (RAMP), Sequence Characterized Amplified Region (SCAR), Simple Primer Amplification Reaction (SPAR), Sequence Related Amplified Polymorphism (SRAP) and Target Region Amplification Polymorphism (TRAP) (Jonah et al., 2011). A number of SSR markers are available in pearl millet and these markers have been used for genetic diversity analysis, linkage and QTL mapping (Gulia, Singh, & Wilson, 2010). Molecular markers systems are efficient tools to detect variations at DNA sequence level in the genome and allow the association of these variations to phenotypic traits which are useful in crossing programmes (Xu, 2010).

SSR markers have been used in many genetic diversity studies in pearl millet (Chandra-Shekara, Prasanna, Bhat, & Singh, 2007; Kapila et al., 2008; Tara Satyavathi et al., 2013), construction of the genetic map (Kannan et al., 2014), identification of cultivars (Kumar, Jacob, Tara Satyavathi, Dadlani, & Kumar, 2016; Neto et al., 2013) and marker assisted selection (Yadav et al., 2011). McBenedict et al. (2016) studied genetic diversity of Namibian pearl millet accessions using SSR markers and reported higher similarity (68 %) among genotypes. In general, the application of SSR markers in genetic diversity analysis have been more effective than other marker systems and it has increased acceptance world-wide (Ramakrishnan, Antony Ceasar, Duraipandiyan, Al-Dhabi, & Ignacimuthu, 2016).

Single Nucleotide Polymorphism (SNPs) are co-dominant markers with high genome coverage, often linked to genes and present in the simplest/ultimate form for polymorphism, and thus they have become very attractive and potential tool for genetic

and breeding study (Jiang, 2013; Suprasanna et al., 2015). The abundance, ubiquity and interspersed nature of SNPs together with the potential of automatic high-throughput analysis, cost effective and quick detectability with a high efficiency for detection of polymorphism make them ideal candidates as molecular markers for construction of high density genetic maps, association analysis of candidate genes, fine mapping of quantitative trait loci, genetic diversity assessment and marker-assisted plant breeding (Jiang, 2013; Sehgal et al., 2012; Suprasanna et al., 2015; Xu, 2010). In addition, SNPs located in known genome regions provide a fast alternative to analyse the fate of agronomical important alleles in breeding populations, thus providing functional markers (Sehgal et al., 2012).

However, high establishment costs, high-quality DNA requirement and high technical/equipment demands limits the application of SNPs in some laboratories and practical breeding programs (Jiang, 2013). Sehgal et al. (2012) used SNP markers in pearl millet in their study of a map based gene associations with assigned functional roles in plant adapted to drought and other abiotic stresses. It was demonstrated that SNPs are useful in identifying candidate genes underlying a major drought tolerance (DT) quantitative trait loci (QTLs). Hu et al. (2015) also reported the successful of SNP markers in genetic diversity assessment of pearl millet accessions collected from Senegal, southern Africa and Asia. Table 1 shows merits and demerits of widely used DNA molecular markers. The application of a given marker system is rely largely on the purposes of the research, available genetic resources, equipment, financial resources and trained personnel's (Jiang, 2013).

Other molecular tools have been developed that can be used for screening of mutants at the gene level such as genome sequencing. Genome sequencing is considered as the 'gold

standard' for mutation detection as it can reveal the exact location of a mutation and its type. Applying this tool to large populations is costly and direct sequencing may not readily detect heterozygous alleles and is rarely used in practical mutation breeding (Pathirana, 2011). However, with next-generation sequencing (NGS) technology, it is now conceivable to sequence candidate amplicons in multiple parallel reactions (SCAMPR) and the costs are comparable to Targeting Induced Local Lesions In Genomes (TILLING) (Pathirana, 2011). TILLING markers have been developed to scan amplicons in mutant populations for the presence of sequence polymorphisms.

It is a reverse genetics approach which has emerged as a robust, high throughput method that can be applied to many species and relies on the detection of sequence alterations in target genes to identify allelic variations among mutant populations (Pathirana, 2011; Roychowdhury & Tah, 2013). It relies on the detection of single-base mismatches in heteroduplexes using endonucleases enzymatic detection method in combination with a sensitive mutation detection instrument i.e. gel electrophoresis or denaturing high-performance liquid chromatography (Pathirana, 2011). TILLING is very efficient for detecting mutations in large (1–2 kb) exon-rich amplicons from target genes. It is less productive when used to screen genes with multiple small exons separated by larger introns, as mutations in introns except those at splice junctions, rarely affect gene function (Pathirana, 2011; Roychowdhury & Tah, 2013).

Table 1 Comparison of most widely used DNA molecular marker systems in plants. Adapted from Jiang, 2013 and Xu, 2010.

Feature and description	**Name of DNA marker system**				
	RFLP P	**RAPD**	**AFLP**	**SSR**	**SN**
Genomic abundance	High	High	High	Moderate to high	Very high
Genomic coverage	Low copy coding region	Whole genome	Whole genome	Whole genome	Whole genome
Expression/inheritance	Co-dominant	Dominant	Dominant / codominant	Co-dominant	Co-dominant
Level of polymorphism	Moderate	High	High	High	High
Type of probes/primers	Low copy/cDNA clones	10 bp random nucleotides	Specific sequence	Specific sequence	Allele-specific primers
PCR-based	Usually no	Yes	Yes	Yes	Yes
Reproducibility/ reliability	High	Low	High	High	High
Amount of DNA required	Large (5 – 50 μg)	Small (0.01 – 0.1 μg)	Moderate (0.5 – 1.0 μg)	Small (0.05 – 0.12 μg)	Small (≥ 0.05 μg)
Quality of DNA required	High	Moderate	High	Moderate to high	High

Feature and description	Name of DNA marker system				
	RFLP P	**RAPD**	**AFLP**	**SSR**	**SN**
Technically demanding	Moderate	Low	Moderate	Low	High
Time demanding	High	Low	Moderate	Low	Low
Ease of use	Not easy	Easy	Moderate	Easy	Easy
Development/start-up cost	Moderate to high	Low	Moderate	Moderate to high	High
Cost per analysis	High	Low	Moderate	Low	Low
Primary application	Genetics	Diversity	Diversity and genetics	All purposes	All purposes

2.7 Grain metabolites/nutrition of pearl millet's putative mutants

Biochemical parameters under this study are the proximate composition, nutritionally valuable minerals, anti-nutrients and the functional properties of the seed flour of pearl millet samples. These parameters may be also qualified to be classified as both primary and secondary metabolites (Roessner & Bowne, 2009). Metabolites such as carbohydrates, lipids, amino acids, vitamins, hormones, flavonoids, phenolics, and glucosinolates are essential for plant growth, development, stress adaptation, and defence. Metabolites determine the nutritional quality of foods, colour, taste, smell, anti-oxidant, anti-hypertension, immune-stimulating, and cholesterol-lowering properties (Mathew & Padmanaban, 2013). Furthermore, unlike genes and proteins, the functions of metabolites subjected to epigenetic regulation and posttranslational modifications. Metabolites serve as direct signatures of biochemical activity and are therefore easier to correlate with phenotypes (Fiehn, 2002; Mathew & Padmanaban, 2013).

Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes (Fiehn, 2002). Therefore, change in biochemical parameters can give a snapshot of the molecular changes that were artificially induced during irradiation mutation of the pearl millet under this study. However, the major disadvantages of biochemical markers are they are limited in number and are influenced by environmental factors or the developmental stage of the plant (El-Nahas et al., 2011). Kiprotich et al. (2015) reported that biochemical traits are useful tool for determining genetic variability in pearl millet and they can contribute to breeding programs and enhance food security. Other researchers conducted on some biochemical traits of sorghum putative mutants showed insignificant mean square values, while the anti-nutrient content showed elevated level in comparison to

the parental lines as a result of induced mutation (Omondi et al., 2012). Therefore, analysis of orphan crops like pearl millet as alternative strategies to curb under nutrition and boost food security is of utmost importance to widen the essential nutrient sources for human beings (Kiprotich et al., 2015).

CHAPTER 3

METHODOLOGY

3.1 Research Design

Genetic and biochemical characterization analyses were performed on grains of 12 pearl millet putative mutant lines and two parental lines of *Okashana 2* and *Kangara* from three Research Stations. To avoid contamination during the pollination period and to ensure the purity of the seeds, each accession was bagged with pollination bag before flowering and seeds were harvested separately.

A stratified sampling technique was applied to select two samples from each putative mutant line grown in three agro-ecological zones (Research Stations). Two samples were randomly selected from the best five higher yielding putative mutants per variety depended on the harvested weight of its grains at each Research Station.

The laboratory study was based on genetic characterizations using SSR markers and the biochemical characterization (proximate composition, nutritionally valuable minerals, anti-nutrients and the functional properties of the seed flour) of the pearl millet's putative mutant germplasms. Proximate and mineral contents were calculated from dry weight values using moisture content of each sample and all analysis were done in triplicates. In accordance with Unam's normative, prior to the undertaking of the research, a student has received the Ethical clearance certificate (APPENDIX A, A1) and the Research permission letter (APPENDIX A, A2).

3.2 Plant materials and sample collection of pearl millet's germplasms

In this study, 12 putative mutant genotypes derived from two locally popular pearl millet varieties, *Okashana* 2 and *Kangara,* and their parental lines were used as a control. Seeds were collected from Oshikoto region (Manheim research station), Omusati region (Omahenene research station) and Kavango East (Bagani research station) during harvesting time (May and June 2015). From each research station, two putative mutant samples of both *Kangara* and *Okashana 2* were collected. In total, fourteen samples *Kangara* derivatives (L5AP18, L7AP1A, R6P1-1, R9P5, L8P5Rep2 and L9P3Rep1), *Okashana 2* derivatives (L3P5, L4P1, L8P12, L10P18, L5P11Rep2 and L10P13Rep1) and the two parental lines of *Kangara* (SDMV 92040) and *Okashana 2* (SDMV 93032) varieties were collected (APPENDIX B, Table B 1).

3.3 Analytical procedures for analyzing of pearl millet's germplasms

Simple Sequence Repeat marker and biochemical parameters analysis were done at the Analytical Service and Product Development (ASPD) Laboratory of the MAWF, Windhoek, Namibia. Pictures of each seed samples were taken and seeds were weighed using the HCB 1002 scale balance (Adam Equipment, Milton Keynes, UK). The seeds were then crushed with a clean and sterile 8010G model blender (Waring Commercial, Torington CT, USA) to obtain homogenous samples. Figure 2 presents the main activities of the flowchart of analytical activities that were performed in this research study.

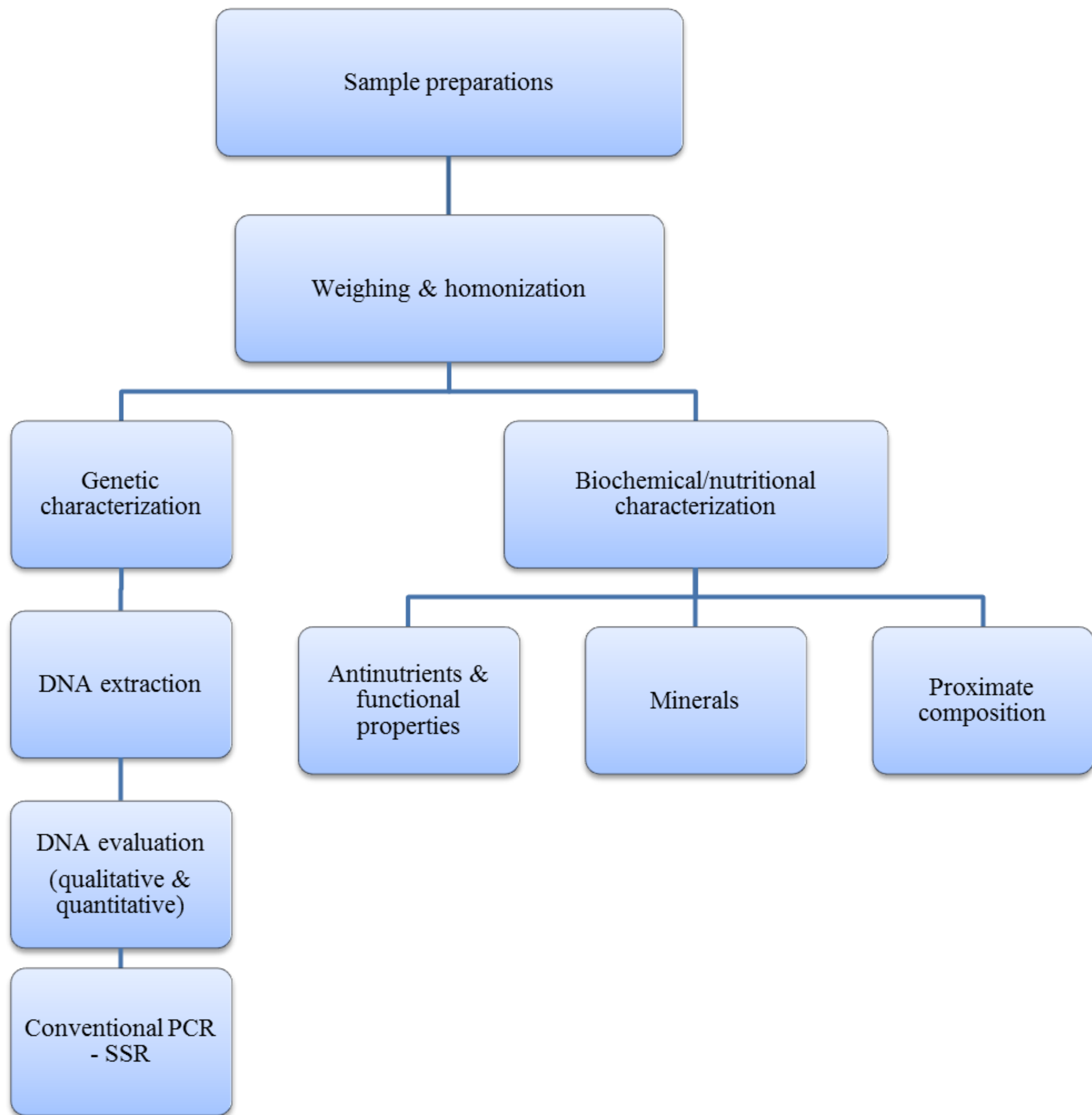


Figure 2 Flowchart of analytical activities performed on each sample.

3.4 Genotypic characterization using SSRs markers

3.4.1 Genomic DNA extraction from pearl millet's grains

Commercial DNAExtractor Clean kit (Eurofins, GeneScan, GmbH, German) for the extraction of DNA from food, feed and grain (Cat. Nos.: 5224700610 and 5224700810) was used to extract genomic DNA from grains (APPENDIX C, C1). From each homogenized sample, 2 g sample was transferred into 15 ml tubes, followed by the addition of 10 ml lysis buffer (100 mM Tris-Cl, PH = 8.0, 10 mM EDTA) and 10 µl of proteinase (20 mg ml^{-1}). The samples were incubated at 60 ^{o}C for 2 h and then centrifuged at 4 000 x g for 10 min. Then, the supernatant were transferred into new clean tube. Add 600 µl of chloroform into the supernatant and the tubes were mixed by vortexing. The aqueous supernatant was transferred into a fresh tube and 2 µl of glycogen solution with 480 µl of 80 % isopropanol were added into the solution followed by thoroughly mixing. Discard the supernatants after incubating the solutions for 20 min at room temperature and centrifuged at 10 000 x g for 15 min. After a repeated cleaning the DNA pellet was eluted with 100 µL 0.1x TE buffer, applied the extracted DNA to a cleaning column following the DNA purification protocol (APPENDIX C, C2) and then stored at -20 ^{o}C.

3.4.2 Qualitative DNA evaluation using Gel electrophoresis

Gel electrophoresis is one of the standard methods used to separate, identify and purify DNA fragments. Samples were tested on 1.0 % agarose gel to check the DNA concentration, size, quality and fidelity (Gulia et al., 2010). Electrophoresis was done according to the protocol presented in APPENDIX C, C3 and C4 and visualized using UV trans-illuminator (Syngene, Cambridge, UK). APPENDIX B, Table B 2 presents the order

of the wells as they appear on the agarose gels i.e. 1 corresponds to M which is a DNA molecular marker and so on.

3.4.3 Quantitative DNA evaluation using Nanodrop spectrophotometer

The concentration and purity of the DNA is importance. Consequently, the concentrations and the purity of the extracted DNA were determined by the ratio of the absorbance at 260 and 280 nm using NanoDrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA). DNA quality is considered as good with an optical density (OD) 260:280 ratio between 1.7 and 2.0 (Gulia et al., 2010). DNA quantification was performed using a NanoDrop 2000c spectrophotometer.

3.4.4 SSRs molecular markers analysis

In this study, 19 selected SSR markers that were synthesized by Integrated DNA Technologies (IDT) were used (Yadav et al., 2007). The selected SSR markers are composed of eight di-, eight tri- and three tetra-nucleotide repeat motifs (Table 2). A total PCR reaction mixture of 25 µL was setup consisting of One Tag 2X Master Mix with Standard Buffer, 0.5 µL (10 µM) each primer (both forward and reverse primer), 6.5 µL of distilled water, 0.2 mM $MgCl_2$ added additional and 20 ng DNA templates.

PCR amplification was performed on an Arktik Thermal Cycler (Thermo Fisher Scientific Oy, Vantaa, Finland) programmed for 35 cycles of 30 s at 94°C (denaturation), annealing at 55 °C for 45 s, extension for 1 minute at 68 °C and ending with 5 minutes at 68 °C after an initial denaturation for 3 minutes at 94 °C.

The PCR products were fractioned on 2 % agarose gels consisting of 3.0 g of agarose (TopVision Agarose, Fermentas) and 3.0 µL of ethidium bromide in 150 ml of 1x Tris-Acetate-EDTA (TAE) buffer. Electrophoresis was performed at 100 volts for 50 minutes at room temperature. Gels were photographed using InGenius LHR UV documentation system (Syngene, Cambridge, UK).

Table 2 SSR primers used in the study including their loci DNA sequences, expected amplicon size and GC content.

Locus	**DNA sequence (5'-3')**	**Expected fragment size (bp)**	**GC content (%)**
Xcump001	F: GCACGAGGCTTATCTGTGTTTC	157	50.0
	R: CAACTCTTGCCTTTCTTGGCCT		50.0
Xcump 002	F: GCACGAGGCAAAATATAAAGGTG	198	43.5
	R: ACGTAGACTTGCACCACCAGA		52.4
Xcump 003	F: CATGCGACGTGGTCTATCTG	118	55.0
	R: GAGAGAGAACCAGCAGCACC		60.0
Xcump 004	F: CACGAGGCTCACTAGGGTTT	113	55.0
	R: ACCCGGGTCTGGTTAGACTT		55.0
Xcump 005	F: GCACGAGGGCCAGATTCTAGAA	164	54.5
	R: CACGGTGATGACACGACATGGT		54.5
Xcump 006	F: GAAATCGGCAGAGGGCAT	100	55.6
	R: CAATGAGTATGTGCACGCTGCA		50.0
Xcump 007	F: GAGGGATTCCAGGCGGTTC	201	63.2
	R: GCGAGGAGCACATTCGATGAA		52.4
Xcump 008	F: GTTGACTACCACTATTATGCTCC	175	43.5
	R: GACCAAGAACTTCATACAATTCAG		37.5
Xcump 009	F: ATCTGATCGTGAGGCCTCAAC	225	52.4
	R: GCCGACCAAGAACTTCATACAAT		43.5
Xcump 010	F: GCTGAACTATTCTGTAAACTTAAC	173	33.3
	R: TATCGAAACGGTACTAAAATCATG		33.3
Xcump 011	F: TGATGGGAACCGAGAGCATGA	196	52.4
	R: TAGCACAGCAATAACATGGCATC		43.5
Xcump 012	F: TGTGATCTGTGGTCTCAGGC	165	55.0
	R: CGTGAAAGCTCTCCAGGACT		55.0
Xcump 013	F: ACCGACAGCAACAAATCCTCC	194	52.4
	R: GCTCTTGTGTGTAGTTGTGCTT		45.5
Xcump 014	F: CTGACCTCTCCTCTCCTTCG	185	60.0
	R: GAGCAGATCCTTGGCCTTCTTG		54.5
Xcump 015	F: GAAGCATAGGAGAGGAGGG	158	57.9
	R: CTTGCTGCTCGGACTTCTCT		55.0
Xcump 016	F: CATTTCTCTCGCCAGTGCTC	250	55.0
	R: ATCTCCAGAACCGAGCGCA		57.9
Xcump 017	F: ATAGCTGGGTGTTGTCTGGC	124	55.0
	R: CCCTGGCGCTTAATTGTAAA		45.0
Xcump 018	F: TGCTTTCTTCCCAACCAGTGG	264	52.4

	R: TGCTGAGTGGGGTGCTGCT		63.2
Xcump 019	F: GGCCTAACTCTCTGTTCTTCTTC	212	47.8
	R: GAGAAGCTAACATTTGGGGCCTA		47.8

F = Forward; R = Reverse

3.5. Biochemical characterization of pearl millet's putative mutant germplasms

3.5.1 Proximate composition analysis

In this study, the official methods of the Association of Official Analytical Chemists (AOAC) were used to determine moisture, crude protein, crude lipid/fat, ash and mineral contents (Horwitz & Latimer, 2006) (APPENDIX D). Total protein for grain was estimated by multiplying the percentages of nitrogen with 5.83 Jones factor (FAO/INFOODS, 2012). Crude fibre, neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Palic (2007) protocol (APPENDIX D). Carbohydrate content was determined by subtracting from 100 the sum percentages of moisture, fat, crude protein, ash and crude fibres (Ijarotimi, Adeoti, & Ariyo, 2013). Energy value was estimated by multiplying the percentages of crude protein, crude lipid/fat, and carbohydrate with the factors of 2.44, 8.37 and 3.57, respectively, proposed by Martin and Coolidge (1978) and cited by Ijarotimi et al. (2013). Furthermore, anti-nutrient of phytate was determined using indirect colorimetric method of Wheeler & Ferrel (1971) while oxalate was determined according to Horwitz & Latimer (2006) protocol (APPENDIX D).

3.5.2 Determination of functional properties of pearl millet's putative mutant flour

Water absorption capacity of the homogenized sample was determined according to the method reported by Beuchat (1977) and cited by Ijarotimi et al (2013) with modification for small samples (APPENDIX D). The bulk density was determined using the method described by Okaka and Potter (1979) as cited by Ijarotini et al. (2013) (APPENDIX D). Swelling power was determined using the method described by Leach et al. (1959) as cited by Ijarotini et al. (2013) with modification for a small sample (APPENDIX D).

3.6 Data analysis

The SSR marker was evaluated for presence/absence (1 = presence and 0 = absence) of a band corresponding to a particular marker comparing to a known molecular weight ladder marker (Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, 2015). The binary data was then entered into Primer-E 5 for Windows (Plymouth Routines in Multivariate Ecological Research) software package (Clarke, 2001) for similarity calculations and cluster analysis. The bands with same mobility are treated as identical bands and a frequency distribution table was created. This is done by giving the band that appeared first above all others an alphabetical code of "A" while the lowest band that appeared last (with lowest amplicon size) given a lower code of letter depending on the number of bands that occur before it. Furthermore, each SSR band was considered as an independent locus, and only distinct, reproducible, and well-resolved fragments were scored visually for each of the 14 genotypes. A locus was considered polymorphic if a consistent band was present in one or more, but not all, individuals of the population. In contrast, qualitative differences in band intensity were not considered. The 14 putative mutants were clustered based on the matrix of Bray-Curtis genetic similarity coefficients

after which a dendrogram was generated using Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) cluster algorithm.

Analysis of variance (ANOVA) for the biochemical traits was conducted using Statistical Package for Social Sciences (SPSS) version 16.0 [or PASW Statistics 18]. The mean, standard error of the mean (SEM) of the samples, Pearson's correlation, coefficient of variation, Fisher's least significant difference, as well as the significant differences determined at $P < 0.05$ of biochemical characters of pearl millet mutants were calculated. All the measurements were made on three separate samples taken from the same sample. Water content in the cereal grains has been analysed and the nutrient contents were then calculated from dry weight values assuming such moisture contents.

CHAPTER 4

RESULTS

4.1 DNA extraction of pearl millet germplasms

Genomic DNA was successfully extracted from all the 14 pearl millet's seed samples, of which two are parental lines (untreated) and 12 are putative mutant lines (gamma-rays treated). DNA extraction protocol used in this study yielded a genomic DNA concentration ranged from 324 to 746 ng/μL and DNA purity ranged from 1.79 to 1.87 (Table 3). Figure 3 revealed the sharp and distinct bands obtained after running the extracted DNA on a 1 % agarose gel, stained with ethidium bromide and visualized with UV light. Although the intensity of the genomic DNA bands are varied, quality DNA was extracted from all genotypes.

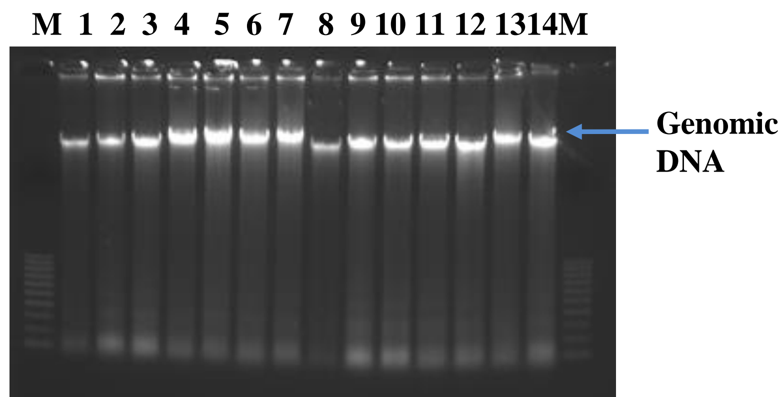


Figure 3 Gel electrophoresis of pearl millet genomic DNA on 1 % agarose stained with 0.5 % ethidium bromide.

Keys: 1 and 8 = *Kangara* and *Okashana 2* controls, 3 – 14 = putative mutants, M = 100 bp DNA ladder

Table 3 Concentration and A260/A280 ratio of extracted genomic DNA of putative mutant pearl millet lines and their respective parental lines.

Sample number	Sample ID	Concentration (ng/µL)	A260/A280
1	OFS	324	1.87
2	L5AP18	405	1.81
3	L7AP1A	397	1.81
4	R6P1-1	486	1.86
5	R9P5	621	1.82
6	L8P5Rep2	669	1.83
7	L9P3Rep1	690	1.84
8	OFS	337	1.87
9	L3P5	623	1.84
10	L4P1	577	1.81
11	L8P12	539	1.79
12	L10P18	522	1.86
13	L5P11R2	493	1.85
14	L10P13R1	746	1.84

Key: 1 and 8 = Kangara and Okashana 2 controls, 3 – 14 = putative mutants.

4.2 SSR PCR marker analysis

Nineteen SSR primer pairs (Table 2) were used to analyse the 14 pearl millet genotypic. A total of 108 SSR fragments were generated from 11 SSR markers, whereas 8 (Xcump007, Xcump011 Xcump013, Xcump014, Xcump015 Xcump016 Xcump017 and Xcump019) markers failed to amplify any fragment on all the pearl millet genotypes (Table 4).

Table 4 Distribution of bands/polymorphisms obtained from 11 SSR markers on 14 pearl millet genotypes.

Bands	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
Total	8	12	5	18	4	1	5	13	12	13	4	13
%	7.4	11.1	4.6	16.7	3.7	1.0	4.6	12.0	11.1	12.0	3.7	12.0
OAS (bp)	±1150	±900	±750	±400	±380	±300	±200	±160	±150	±120	±100	±90

Keys: OAS = Observed amplicon size

There was only Xcump006 marker with a fragment size of 300 bp specific rare allele observed in 14 samples and there were no other specific bands unique to a single genotype observed in this analysis. However, few bands were shared among certain genotypes and not with other genotypes when different primers were used. Figure 4 below shows one of the PCR amplification of the 11 SSR primers that successful amplified the extracted DNA under this study.

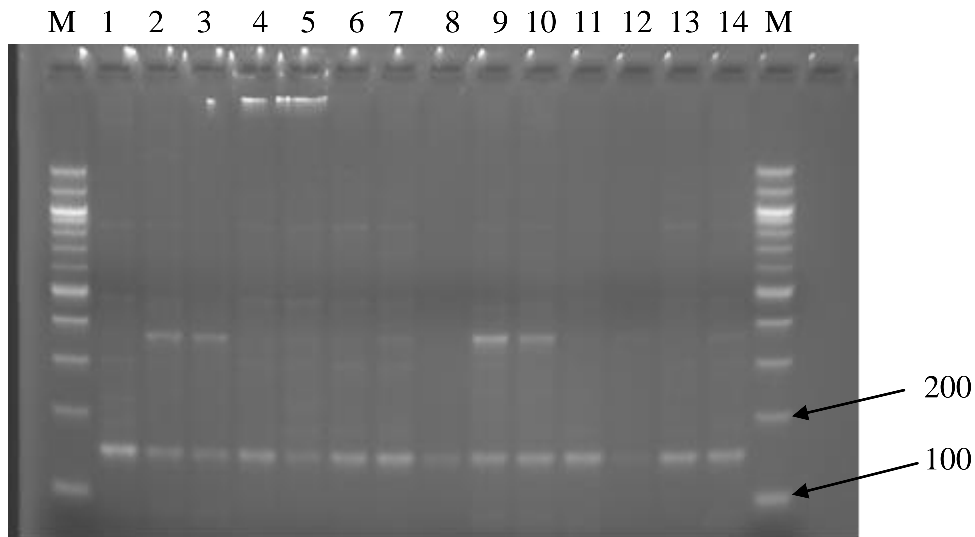


Figure 4 PCR products of Xcump006 primer pairs corresponding to 14 pearl millet genotypes.

4.3 SSR genetic diversity calculations

4.3.1 Polymorphism information content (PIC)

Polymorphism information content (PIC) for each SSR marker was determined as described in Senior, Murphy, Goodman, & Stuber (1998)

PIC = $1 - \sum f_i^2$, where f_i is the frequency of the ith allele.

PIC provides an estimate of the discriminatory power of a marker by taking into account not only the number of alleles at a locus, but also the relative frequencies of those alleles in the population under study (Senior et al., 1998). PIC values ranged from 0 (monomorphic) to 1 (highly polymorphic), with many alleles in equal frequencies (Ali et al., 2008). In this study, PIC values ranged from 0.14 (Xcump 008 and Xcump 009) to 0.87 (Xcump 012) with a mean of 0.38. Only two markers (Xcump003 and Xcump012) had a PIC value of

more than 0.5 (Table 5). Of all the tested SSR markers, 64 % of the markers were polymorphic and the remaining markers were monomorphic.

Table 5 Number of bands score and Polymorphic Information Content (PIC) for the 11 SSR markers tested on 14 pearl millet genotypes.

Locus	Number of bands	PIC
Xcump 003	28.0	0.55
Xcump 004	20.0	0.24
Xcump 006	16.0	0.45
Xcump 008	13.0	0.14
Xcump 009	13.0	0.14
Xcump 012	5.0	0.87
Xcump 018	12.0	0.27
Mean	**15.3**	**0.38**

4.3.2 Shannon-Wiener Diversity Index (H')

Shannon-Wiener diversity index (H') is defined as a measure of intra-population genetic diversity (Nghishikungu-Horn, 2007).

$H' = -\sum(Pi)(\ln Pi)$, where *Pi* is proportion of the *i*th allele in the population while *ln* is the natural logarithm of *Pi.*

The calculated Shannon Wiener index was H' = 2.589 (Table 6). Values of this index above 3.5 are considered indicators of high species richness and diversity, and values below 1.5 are considered indicators of low species richness and evenness (Magurran, 1988).

Table 6 Shannon-Wiener Diversity Index (H') calculated for 14 pearl millet genotypes using 11 SSR markers

Sample ID	Number of bands	Proportion (Pi)	ln(pi)	Pi*ln(pi)
KFS	11	0.102	-2.284	-0.233
L5AP18	10	0.093	-2.380	-0.221
L7AP1A	11	0102	-2.284	-0.233
R6P1-1	9	0.083	-2.485	-0.206
R9P5	6	0.056	-2.890	-0.162
L8P5Rep2	7	0.065	-2.736	-0.178
L9P3Rep1	7	0.065	-2.736	-0.178
Sub-total	**61**	**0.566**	**-17.795**	**-1.411**
OFS	6	0.056	-2.890	-0.162
L3P5	10	0.093	-2.380	-0.221
L4P1	10	0.093	-2.380	-0.221
L8P12	4	0.037	-3.196	-0.122
L10P18	4	0.037	-3.196	-0.122
L5P11R2	7	0.065	-2.736	-0.178
L10P13R1	6	0.056	-2.890	-0.162
Sub-total	**47**	**0.437**	**-19.668**	**-1.188**
Total	**108**	**1.000**	**-37.463**	**-2.589**
H' = -∑Pi(lnPi) = 2.589				

Key: KFS – *Kangara* foundation seeds parental line,OFS – *Okashana 2* foundation seeds parental line

4.3.3 Simpson's Index of Diversity

Simpson's diversity index (D) is a measure of diversity, which takes into account both species richness and evenness of abundance among the species (Magurran, 1988).

The Simpson's diversity Index (D) = ∑ni(ni-1)/N(N-1), where N is the total number of organisms in the survey while ni is the number of individuals for each species.

The calculated Simpson's Diversity Index calculated in this study was D = 0.93 (Table 7). The value of D ranges from 0 to 1, in which 0 represents infinite diversity and 1 signifies no diversity, the bigger the value the lower the diversity.

Table 7 Simpson Diversity Index (D) calculated for 14 pearl millet genotypes using 11 SSR markers

Sample ID	Number of bands (n)	N(N-1)
KFS	11	110.00
L5AP18	10	90.00
L7AP1A	11	110.00
R6P1-1	9	72.00
R9P5	6	30.00
L8P5Rep2	7	42.00
L9P3Rep1	7	42.00
Sub-total	**61**	**496**
OFS	6	30.00
L3P5	10	90.00
L4P1	10	90.00
L8P12	4	12.00
L10P18	4	12.00
L5P11R2	7	42.00
L10P13R1	6	30.00
Sub-total	**47**	**306**
Total	**108**	**802.00**

Key: KFS – *Kangara* foundation seeds parental line, OFS – *Okashana 2* foundation seeds parental line

4.3.4 Cluster analysis

Cluster analysis generated by UPGMA- using Bra-Curtis similarity level revealed a genetic similarity level of 51.8 % (Figure 5). At the level of 51.8 %, the 14 pearl millet genotypes were divided into two main clusters labelled I and II. The first cluster (I) comprises the *Okashana 2* parental line (OFS) that is distinguished as a separate phenetic line. The second cluster (II) split into two subgroups at the similarity level of 61 %. The first subgroup labelled ‘a’ comprises three genotypes, at the level of 75 % the putative mutant genotype L10P18 which then grouped with the R9P5 and L8P12 that are grouped together at the level of 76 %. At the similarity level of 73 %, the second subgroup labelled ‘b’ is split further into two sub-clusters each comprises of five genotypes.

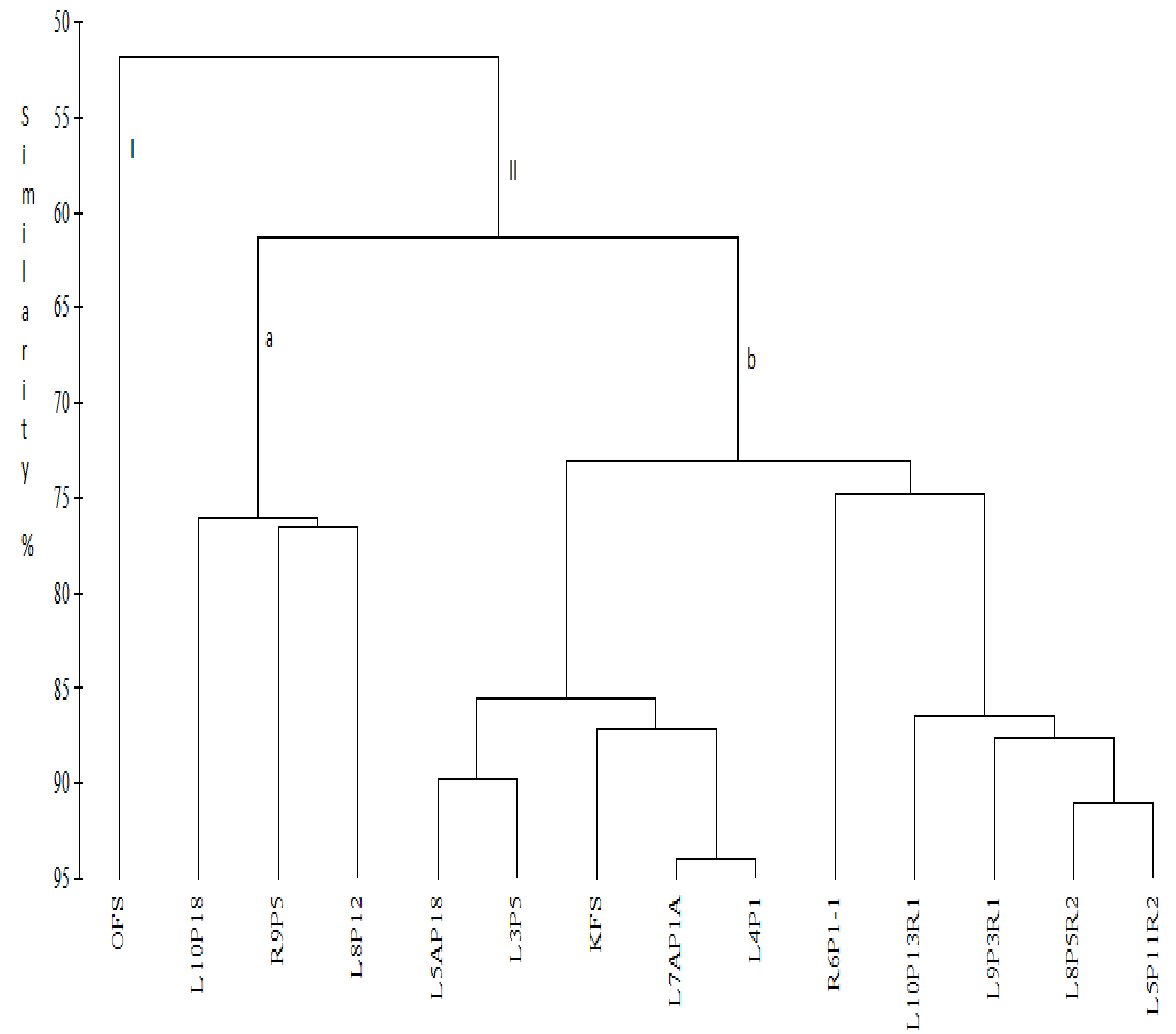


Figure 5 UPGMA similarity phenogram cluster generated from seven SSR primers of 14 pearl millet genotypes.

4.4 Biochemical characterization of pearl millet putative mutant germplasms

4.4.1 Proximate composition analysis

Proximate composition analysis of 14 pearl millet mutant lines derived from *Kangara* and *Okashana 2* parental varieties is presented in Table 8 and Table 9, respectively. Among *Kangara* derived mutant lines, moisture content (%) ranged from 5.98 (L10P1 mutant line) to 8.88 (*Kangara (KFS)* parental variety) while among *Okashana* 2 derivatives, the value ranged from 5.75 (L10P18) to 10.00 (L5P11Rep2). Similarly, the ash percentage ranged from 1.77 to 3.18 among *Kangara* derivatives as compare to 1.87 of parental line. The value was higher for *Okashana* derivatives that ranged from 1.91 to 5.16 within the tested samples and 1.94 for *Okashana 2* (OFS) parental variety.

However, the crude proteins (%) among *Kangara* derivatives ranged from 10.13 (L5AP18) to 14.54 (R9P5) as compare to 11.71 for KFS. Among *Okashana* derivatives, crude protein ranged from 12.04 (L8P12) to 13.93 (L5P11Rep2) compare to 11.24 for OFS parental control. Percent of crude fibre variation among *Kangara* derivatives ranged from 2.51 to 4.70 with the value 2.51 for the KFS parental control. However, among *Okashana* derivatives the variation varied from 2.97 to 4.43 as compared to 4.25 for the parental control. Other major proximate composition indicators of fat (%) has spanned from 5.25 to 5.74 among *Kangara* mutants and with a value of 6.20 for the parental control (KFS). Little bit higher value ranged from 5.18 to 6.36 percent (L8P12) was recorded among *Okashana* mutant lines and the OFS parental control scored a value of 6.33.

Carbohydrates content (%) arrayed from 71.58 (L8P5Rep1) to 75.61 (L5AP18) among *Kangara* lines as compared to 74.05 of KFS parental control. Similar values ranged from 66.84 to 74.23 and 74.50 for OFS parental control was recorded among *Okashana* derivatives. The energy (kkal) level of *Kangara* mutants ranged from 319.39 (L5AP1A) to

328.57 (R6P1-1) as compared to 329.98 of *Kangara* parental line. However, energy level ranged from 307.52 (L5P11Rep2) to 330.13 (L8P12) among *Okashana 2* samples as compared to 331.47 of *Okashana 2* parental control line (OFS.

Table 8 Proximate composition (%) of *Kangara* pearl millet putative mutants

Genotype	**Parameter**										
	Moisture	**Ash**	**OM**	**CP**	**Protein**	**CF**	**ADF**	**NDF**	**Fat**	**Carb**	**Energy**
KFS	8.88	1.87	91.12	11.71	10.92	4.47	8.08	36.29	6.20	74.05	329.98
L5AP18	6.35^{a}	2.23^{a}	93.65^{a}	10.13^{a}	9.45^{a}	4.20^{a}	8.97^{a}	28.47^{a}	5.74^{a}	75.61^{a}	327.60^{a}
L7AP1A	8.15^{a}	3.18^{a}	91.85^{a}	10.56^{a}	9.85^{a}	4.7^{a}	8.19^{a}	30.10^{a}	5.72^{a}	72.92^{b}	319.39^{a}
R6P1-1	6.74^{a}	1.77^{b}	93.26^{a}	12.69^{a}	11.84^{a}	3.83^{a}	8.73^{a}	30.29^{a}	5.56^{a}	74.50^{a}	328.57^{a}
R9P5	5.98^{a}	1.99^{b}	94.02^{a}	14.54^{a}	13.56^{a}	2.51^{a}	8.43^{a}	26.10^{a}	5.25^{a}	73.84^{b}	328.26^{a}
L8P5Rep2	6.18^{a}	2.12^{a}	93.82^{a}	14.5^{a}	13.55^{a}	4.21^{a}	20.01^{a}	30.59^{a}	5.57^{a}	71.58^{a}	323.34^{a}
L9P3Rep1	6.52^{a}	2.59^{a}	93.48^{a}	13.94^{a}	13.00^{a}	3.29^{a}	10.63^{a}	28.56^{a}	5.34^{a}	72.41^{b}	322.76^{a}
Mean	6.97 ± 0.42	2.25 ± 0.19	93.03 ± 0.42	12.58 ± 0.69	11.74 ± 0.65	3.89 ± 0.29	10.43 ± 1.63	30.06 ± 1.19	5.63 ± 0.12	73.56 ± 0.51	325.70 ± 1.47
CV	14.64	20.16	1.10	13.51	13.54	18.07	38.24	9.71	5.12	1.71	1.11
LSD	0.200	0.127	0.200	0.700	0.653	0.086	0.098	0.283	0.105	0.786	1.457

Key: KFS – *Kangara* foundation seeds parental line; OM – organic matter; CP - crude protein; CF – crude fibre; CV – coefficient of variation; LSD- Fisher's least significant figure. Values are means while ± SEM are standard error of the mean (SEM) for n = 3 of triplicate samples, means with an 'a' superscript in the same row show significant difference ($P < 0.05$) while with an 'b' superscript in the same row show no significant difference ($P > 0.05$)

Table 9 Proximate composition (%) of *Okashana 2* pearl millet putative mutants

Genotype	Parameter										
	Moisture	**Ash**	**OM**	**CP**	**Protein**	**CF**	**ADF**	**NDF**	**Fat**	**Carb**	**Energy**
OFS	10.62	1.94	89.38	11.24	10.49	4.25	8.53	29.78	6.33	74.50	331.47
L3P5	6.67^{a}	1.91^{b}	93.33^{a}	13.14^{a}	12.26^{a}	4.43^{a}	9.83^{a}	32.75^{a}	5.78^{a}	72.97^{b}	326.30^{a}
L4P1	7.33^{a}	1.96^{b}	92.67^{a}	12.99^{a}	12.11^{a}	3.77^{a}	7.99^{a}	32.65^{a}	5.62^{a}	73.85^{b}	327.60^{a}
L8P12	5.84^{a}	2.03^{b}	94.16^{a}	12.04^{a}	11.12^{a}	4.21^{b}	8.34^{a}	32.89^{a}	6.36^{b}	73.45^{b}	330.13^{a}
L10P18	5.75^{a}	4.25^{a}	94.25^{a}	13.44^{a}	12.54^{a}	3.63^{a}	10.38^{a}	33.14^{a}	5.18^{a}	69.49^{a}	310.32^{a}
L5P11Rep2	10.00^{a}	5.16^{a}	90.00^{a}	13.93^{a}	12.99^{a}	3.65^{a}	8.38^{a}	28.47^{a}	5.77^{a}	66.84^{a}	307.52^{a}
L10P13Rep1	6.83^{a}	2.19^{a}	93.17^{a}	13.38^{a}	12.48^{a}	2.97^{a}	7.00^{a}	26.58^{a}	5.19^{a}	74.23^{a}	326.27^{a}
Mean	7.58 ± 0.74	2.78 ± 0.51	92.42 ± 0.74	12.88 ± 0.35	12.00 ± 0.33	3.84 ± 0.19	8.64 ± 0.43	30.89 ± 0.99	5.75 ± 0.18	61.70 ± 1.10	322.80 ± 3.67
CV	23.88	44.83	1.96	6.65	6.78	12.03	12.16	7.86	7.68	4.35	2.78
LSD	0.310	0.094	0.310	0.731	0.681	0.092	0.119	0.407	0.281	0.925	1.794

Key: OFS – *Okashana 2* foundation seeds parental line; OM – organic matter; CP - crude protein; CF – crude fibre; CV - coefficient of variation; LSD- Fisher's least significant figure. Values are means ± standard error of the mean (SEM) for n = 3, means with an 'a' superscript in the same row show significant difference (P < 0.05) while with an 'b' superscript in the same row show no significant difference (P > 0.05)

4.4.2 Functional and anti-nutrients properties of pearl millet's putative germplasms

Functional properties and anti-nutrients analysis of 12 pearl millet mutant lines derived from *Kangara* and *Okashana 2* parental varieties are presented in Table 10 and Table 11 respectively. Among *Kangara* derivatives, the percentage of water absorbance capacity (WAC) ranged from 253.50 (R9P5) to 327.00 (L9P3Rep2) as compared to the parental control line (KFS) at 269.33. However, *Okashana 2* parental control line (OFS) was 252.33 with the mutant derivatives ranged from 206.67 (L10P13Rep1) to 303.17 (L3P5). Among *Kangara* varieties, the bulk density (g/cm^3) observed ranged from 0.80 in KFS parental line to 0.91 in R6P1-1 and the values were significantly higher among *Okashana 2* derivatives ranged from of 0.83 (L8P12) to 0.98 in L2P11R2 of the tested samples.

Table 10 Functional and anti-nutrient properties of *Kangara* pearl millet putative mutants.

Genotype	Parameter				
	Water absorbance capacity (%)	Bulk density (g/cm3)	Swelling capacity (g/g)	Phytic acid (%)	Oxalate (mg/100 g)
KFS	269.33	0.80	4.32	6.21	20.0
L5AP18	299.33^a	0.82^a	4.65^a	6.56^a	26.2^a
L7AP1A	307.67^a	0.90^b	4.63^a	5.98^a	22.4^b
R6P1-1	261.00^b	0.91^b	3.96^a	6.00^a	12.9^a
R9P5	253.50^b	0.81^a	4.03^a	6.26^b	18.3^b
L8P5Rep2	297.17^a	0.83^a	4.47^a	5.87^a	25.0^b
L9P3Rep1	327.00^a	0.86^b	4.98^a	6.63^a	13.9^a
Mean	287.86 ± 10.22	0.85 ± 0.02	4.43 ± 0.14	6.22 ± 0.11	19.81 ± 1.95
CV	8.70	4.79	7.55	4.36	24.09
LSD	19.279	0.045	0.115	0.081	5.609

Key: KFS – *Kangara* foundation seeds parental line; CV – coefficient of variation; LSD- Fisher's least significant figure. Values are means while ± SEM are standard error of the mean (SEM) for n = 3 of triplicate samples, means with an 'a' superscript in the same row show significant difference (P < 0.05) while with an 'b' superscript in the same row show no significant difference (P > 0.05)

However, the swelling power (g/g) among *Kangara* derivatives ranged from 3.96 (R6P1-1) to 4.98 (L9P3Rep1) while the value of the control was 4.32. Among *Okashana* derivatives

the value ranged from 3.90 (L8P12) to 4.65 (L3P5) as compared to 4.13 (OFS) parental control.

Table 11 Functional and anti-nutrient properties of *Okashana 2* pearl millet putative mutants

Genotype	Parameter				
	Water absorbance capacity (%)	**Bulk density (g/cm3)**	**Swelling capacity (g/g)**	**Phytic acid (%)**	**Oxalate (mg/100 g)**
OFS	252.33	0.84	4.13	6.26	33.7
L3P5	303.17[a]	0.91[a]	4.65[a]	6.37[b]	25.0[a]
L4P1	276.00[a]	0.92[a]	4.39[a]	6.10[a]	25.0[a]
L8P12	207.33[a]	0.83[b]	3.90[a]	6.95[a]	12.3[a]
L10P18	265.50[b]	0.92[a]	4.20[b]	6.41[a]	22.4[a]
L5P11Rep2	217.67[a]	0.98[a]	4.01[b]	6.21[b]	28.6[a]
L10P13Rep1	206.67[a]	0.96[a]	4.05[b]	6.10[a]	21.4[a]
Mean	246.95 ± 14.17	0.91 ± 0.02	4.19 ± 0.10	6.34 ± 0.11	24.06 ± 2.50
CV	14.05	5.72	5.64	4.28	25.50
LSD	14.669	0.029	0.134	0.111	4.749

Key: OFS – *Okashana 2* foundation seeds parental line; CV – coefficient of variation; LSD- Fisher's least significant figure. Values are means while ± SEM are standard error of the mean (SEM) for n = 3 of triplicate samples, means with an 'a' superscript in the same row show significant difference ($P < 0.05$) while with an 'b' superscript in the same row show no significant difference ($P > 0.05$)

The anti-nutrient phytate (%) values varied from 5.87 (L8P5Rep2) to 6.63 (L9P3Rep1) among *Kangara* mutants and the value for the parental line was at 6.21. The phytate % values on *Okashana 2* derivatives varied from 6.10 (L4P1) to 6.95 (L8P12) with a value of 6.26 on the control genotype. The regression equation within the range of the standard sample concentration of 0 - 40 % was Y = 0.0816x - 0.1993 with a good linearity (R^2 = 0. 9964). On the other hand, oxalate composition (mg/100 g) indicators spanned from 12.9 (R6P1-1) to 26.2 (L5AP8) in *Kangara* derivatives as compared to 20.00 (*Kangara* control), while the value ranged from 12.3 (L8P12) to 28.6 (L5P11Rep2) among *Okashana 2* mutants as compared to the *Okashana 2* control at 33.7 value.

4.4.3 The minerals content of pearl millet's putative mutant germplasms

Five major and two trace mineral elements analysis of the 12 pearl millet mutant lines that were derived from *Kangara* and *Okashana 2* parental varieties are presented in Table 12 and Table 13, respectively. The most abundant macro element (mg/100 g) was potassium, while phosphorus is the less abundant macro-element (mg/100 g). The potassium value ranged from 237.3 (L8P5Rep2) to 362.3 (L5AP18) among *Kangara* derivatives, with 331.4 value for KFS. Among the *Okashana 2* derived mutants, however, the value ranged from 295.7 (L3P5) to 368.5 (L10P18) with parental control (OFS) value of 367.3. Among the *Okashana 2* derivatives, the phosphorous values arrayed from 2.8 (L8P12) to 154.7 (L4P1) with a value 147.7 of the parental control (OFS). While, among *Kangara* samples, calcium was the less abundant (mg/100g) with values ranging from 8.5 (R9P5) to 34.5 (L5AP18) at $P < 0.05$ as compared to 15.7 of the control (KFS).

Table 12 Mineral composition of Kangara pearl millet putative mutants (mg/100 g calculated on dry matter).

Genotype	**Parameter**						
	Ca	**K**	**Mg**	**Na**	**P**	**Fe**	**Zn**
KFS	15.7	331.4	73.5	140.4	32.6	7.9	5.7
L5AP18	34.5^{a}	362.3^{b}	66.2^{a}	128.6^{b}	40.2^{b}	10.4^{a}	6.5^{a}
L7AP1A	20.0^{a}	305.2^{b}	69.3^{b}	125.6^{a}	57.7^{a}	6.6^{a}	5.9^{b}
R6P1-1	20.0^{a}	362.1^{b}	71.1^{b}	141.4^{b}	75.1^{a}	7.1^{a}	6.9^{a}
R9P5	8.5^{a}	255.3^{a}	54.2^{a}	119.1^{a}	100.3^{a}	5.9^{a}	4.8^{a}
L8P5Rep2	11.0^{a}	237.3^{a}	59.7^{a}	115.5^{a}	99.1^{a}	6.7^{a}	5.1^{a}
L9P3Rep1	21.4^{a}	330.2^{b}	66.7^{b}	118.3^{a}	128.0^{a}	6.0^{a}	5.3^{a}
Mean	18.73 ± 3.21	311.97 ± 18.64	65.81 ± 2.55	126.99 ± 3.97	76.14 ± 13.21	7.23 ± 0.59	5.74 ± 0.29
CV	41.98	14.63	9.48	7.66	42.50	19.89	12.20
LSD	3.569	21.585	6.550	6.792	8.858	0.635	0.362

Key: KFS – *Kangara* foundation seeds parental line; CV – coefficient of variation; LSD- Fisher's least significant figure. Values are means while ± SEM are standard error of the mean (SEM) for n = 3 of triplicate samples, means with an 'a' superscript in the same row show significant difference ($P < 0.05$) while with an 'b' superscript in the same row show no significant difference ($P > 0.05$.

Among the trace elements analysed, iron is more abundant (mg/100 g) in both *Kangara* and *Okashana 2* derivatives. Values ranged from 5.9 (R9P5) to 10.4 (L5AP18) as compared to KFS at 7.9. Among *Okashana 2* derivatives, the values ranged from 4.4 (L5P11Rep2) to 6.0 (L10P18) as compared to 7.0 of OFS parental control line.

Table 13 Mineral composition of *Okashana 2* pearl millet putative mutants (mg/100 g calculated on dry matter).

Genotype	**Parameter**						
	Ca	K	Mg	Na	P	Fe	Zn
OFS	23.5	367.3	73.5	135.6	147.7	7.0	5.7
L3P5	19.3^{a}	295.7^{a}	58.9^{a}	123.2^{a}	138.6^{a}	4.6^{a}	5.1^{a}
L4P1	16.2^{a}	303.2^{a}	59.4^{a}	127.1^{a}	154.7^{a}	5.1^{a}	5.5^{b}
L8P12	16.3^{a}	313.0^{a}	57.4^{a}	128.0^{a}	2.8^{a}	4.5^{a}	3.7^{a}
L10P18	21.9^{b}	368.5^{b}	78.9^{a}	126.2^{a}	5.0^{a}	6.0^{a}	4.2^{a}
L5P11Rep2	21.5^{b}	309.6^{a}	72.2^{b}	151.5^{a}	9.3^{a}	4.4^{a}	4.1^{a}
L10P13Rep1	15.0^{a}	332.8^{b}	71.9^{b}	143.1^{a}	18.6^{a}	5.0^{a}	3.4^{a}
Mean	19.10 ± 1.25	327.16 ± 11.36	67.46 ± 3.27	133.53 ± 3.94	68.10 ± 28.01	5.23 ± 0.36	4.53 ± 0.34
CV	16.08	8.51	11.87	7.22	100.76	16.82	18.46
LSD	2.365	13.591	3.429	5.539	2.899	0.572	0.278

Key: OFS – *Okashana 2* foundation seeds parental line; CV – coefficient of variation; LSD- Fisher's least significant figure. Values are means while ± SEM are standard error of the mean (SEM) for n = 3 of triplicate samples, means with an ‘a’ superscript in the same row show significant difference (P < 0.05) while with an ‘b’ superscript in the same row show no significant difference (P > 0.05).

4.5 Correlation analysis of SSR and biochemical markers of pearl millet putative mutant

Pearson's correlation analysis between SSR and biochemical markers of pearl millet putative mutants ranged from -0.44 (between SSR and CP) to 0.70 (between SSR and Zinc) with the mean r = |0.31| (Table 14). Coefficients of correlation estimated between the mineral nutrient contents and proximate composition ranged from -0.63 (between Ca and protein) to 0.61 (between Na and moisture) compared to the mean r = |0.26|. Between the proximate composition and functional and anti-nutrients properties, it ranged from -0.55 (between energy and BD) to 0.58 (between ash and BD) compared to the mean r = |0.21|. Coefficients of correlation estimated between the mineral nutrient contents and functional and anti-nutrients properties ranged from -0.66 (between WAC and Na) to 0.54 (between phytate and Ca) compared to the mean r = |0.29|.

Among the proximate composition biochemical markers, coefficients of correlation ranged from -1.0 (between moisture and OM) to 0.92 (between CH and energy) with the mean r = |0.46|. Coefficients of correlation estimated among the mineral contents biochemical markers ranged from -0.46 (between Na and P) to 0.73 (between Ca and K) compare to the mean r = |0.54|. Coefficients of correlation estimated among the functional and anti-nutrients properties biochemical markers ranged -0.33 (between phytate and oxalate) to 0.91 (between WAC and SC) with the mean r = |0.58|. Highest negative correlation was observed between moisture and OM (r = -1.0) followed by between energy and ash (r = -0.96), on the other hand, the highest positive correlation was observed between CH and energy (r = 0.92), followed by SC and WAC (r = 0.91).

Table 14 Correlation analysis of SSR and biochemical markers of pearl millet putative mutants

	SSR bands	*Moisture*	*Ash*	*OM*	*CP*	*Protein*	*CF*	*ADF*	*NDF*	*Fat*	*CH*	*Energy*	*Ca*	*K*	*Mg*	*Na*	*P*	*Fe*	*Zn*	*WAC*	*BD*	*SC*	*Phytate*	*Oxalate*
SSR bands	1																							
Moisture	0.237947	1																						
Ash	-0.25212	0.248313	1																					
OM	-0.23795	-1	-0.24831	1																				
CP	-0.44267	-0.3036	0.185198	0.3036	1																			
Protein	-0.43181	-0.2973	0.18664	0.297304	0.999733	1																		
CF	0.493352	0.307063	-0.09063	-0.30706	-0.65985	-0.65886	1																	
ADF	-0.12545	-0.29087	-0.03842	0.290875	0.403915	0.409599	0.163155	1																
NDF	0.251821	0.054381	-0.10665	-0.05438	-0.23502	-0.23932	0.64236	0.058848	1															
Fat	0.1586	0.525926	-0.23165	-0.52593	-0.57653	-0.58504	0.697489	-0.1502	0.494943	1														
CH	0.332559	-0.17796	-0.89307	0.177958	-0.49951	-0.49932	0.097273	-0.24308	-0.02448	0.201717	1													
Energy	0.214604	-0.09612	-0.96547	0.096122	-0.31722	-0.32097	0.106311	-0.13105	0.084588	0.392105	0.916564	1												
Ca	0.214719	0.191503	0.239719	-0.1915	-0.63936	-0.63471	0.35534	-0.24633	-0.03154	0.181808	0.065309	-0.15107	1											
K	-0.03268	0.227682	0.113097	-0.22768	-0.53609	-0.53447	0.135535	-0.50848	0.130831	0.144199	0.173857	-0.00531	0.727448	1										
Mg	-0.03358	0.458935	0.47631	-0.45893	-0.26743	-0.25877	0.124222	-0.23364	0.120307	-0.03051	-0.26341	-0.42041	0.440614	0.740983	1									
Na	0.061225	0.610206	0.303744	-0.61021	-0.18738	-0.18718	0.00807	-0.53177	-0.00903	0.24425	-0.17202	-0.1626	0.21143	0.479031	0.598715	1								
P	0.283738	0.102034	-0.49017	-0.10203	0.144077	0.152112	0.009646	0.198204	-0.05947	0.001156	0.325589	0.414317	-0.12787	-0.24708	-0.40867	-0.46081	1							
Fe	0.407843	0.020241	-0.26052	-0.02024	-0.59323	-0.58263	0.29785	0.099996	-0.00073	0.119006	0.48199	0.286598	0.538233	0.364247	0.240933	-0.05908	-0.03298	1						
Zn	0.700046	0.149646	-0.37078	-0.14965	-0.49043	-0.47875	0.413913	0.036175	0.11457	0.168841	0.477178	0.3513	0.402633	0.241725	0.060648	-0.09445	0.429826	0.716259	1					
WAC	0.357652	-0.46313	-0.14909	0.46313	-0.12459	-0.12522	0.243053	0.334997	0.230805	-0.11845	0.147675	0.046006	0.094377	-0.19963	-0.32634	-0.66296	0.195039	0.301372	0.40555	1				
BD	-0.31047	0.056408	0.577151	-0.05641	0.181672	0.17457	-0.20877	-0.2973	-0.15023	-0.22588	-0.50241	-0.54999	0.018304	0.189469	0.306016	0.514026	-0.46481	-0.58645	-0.50819	-0.28196	1			
SC	0.304134	-0.30915	-0.06489	0.309152	-0.17171	-0.17292	0.193589	0.224235	0.117889	-0.08406	0.115721	0.005473	0.204624	-0.11821	-0.23198	-0.5451	0.15482	0.235116	0.21831	0.905819	-0.16729	1		
Phytate	-0.14256	0.330603	-0.01678	-0.3306	-0.24615	-0.24257	-0.07893	-0.27493	-0.09401	0.175987	0.159815	0.131626	0.536059	0.52599	0.230135	-0.03239	0.380207	0.248379	0.164885	-0.11696	-0.29446	0.056737	1	
Oxalate	0.189096	-0.16392	0.261539	0.163916	0.046572	0.043438	0.289362	0.131818	0.226811	0.056276	-0.33741	-0.31134	0.044168	-0.37643	-0.33305	-0.06691	-0.2003	-0.27785	-0.32874	0.036356	0.166585	0.079965	-0.32867	1

Key: OM - organic matter; CP – crude protein; CF - crude fibre; CH – carbohydrates; WAC- water absorbance capacity; BD –bulk density; SC- swelling capacity

CHAPTER 5
DISCUSSIONS

5.1 Genotypic characterization of pearl millet's putative mutant using SSR markers

5.1.1 DNA extraction of pearl millet germplasms

Extraction of pearl millet genomic DNA was achieved by the use of a commercial kit of DNA Extractor (Eurofins, Freiburg, Germany) with a hand-on time less than 1 hour. The results (DNA concentration, purity and fragmentation state) indicated that it is possible to use this method to extract genomic DNA from pearl millet's grains. Similar extraction was done on soyabean using same kit which yielded a genomic DNA concentration ranging from 190 to 330 ng/µL and nucleic acid purity ranging from 1.78 to 1.81 (Stefanova, Taseva, Georgieva, Gotcheva, & Angelov, 2013). Nweke, Osuji, Mairo, & Onyenekwe (2014) reported higher genomic DNA concentration (1500 to 1700 ng per extraction) and nucleic acid purity ranging from 1.7 to 1.9 using pearl millet. The genomic DNA concentration found in this study (between 324 and 746 ng/µL) is lower than the previous studies but it was sufficient for various downstream applications (Liang, Deng, Wang, & Xu, 2016).

The DNA concentration variation reported could be due to the difference in initial sample weight used in the two studies. Also, the downsized of available genomic DNA by transferring 0.8 mL from the supernatant for further processing employed in this study may have contributed for the further variation observed. Furthermore, the DNA purity observed in this study concurred with earlier studies might point to the possibility of contribution by the different matrix used. Therefore, the DNA extraction protocol (appendix B) used in this study was suitable for the purpose as it has exhibited an average DNA concentration, fragmentation state and purity that is considered good (Gulia et al., 2010). The quantity

and purity of DNA are the most critical factors for PCR analysis, therefore suitable extraction methods should be applied (Querci, Jermini, & Van den Eede, 2006).

5.1.2 SSR analysis

PCR amplification of the SSR markers in this study had produced clear and scorable bands following ethidium bromide staining on agarose gel electrophoresis. As reported by Yadav et al. (2007), only 68 % (11 SSR) of the markers were successful to amplify scorable bands and the remaining loci were not successful to amplify any fragments on the tested pearl millet genotypes. Although equal numbers of loci were reported to be amplified in both studies, three of the markers (Xcump007, Xcump016 and Xcump017) that were successful in the above study could not amplify any fragment in this study. However, in this study locus Xcump004, Xcump006 and Xcump012 were successful though failed in early study. The variation may not only be due to variation in pearl millet sample used in both studies but also due to variation in intron lengths that may affect the PCR conditions and may not be appropriate for consistent amplification of fairly large DNA fragments (Yadav et al., 2007).

The mean genetic diversity (0.38) observed in this study was lower than the genetic diversity value reported by previous studies (Ali et al., 2008; Chandra-Shekara et al., 2007; Senior et al., 1998; Tara Satyavathi et al., 2013) . The lower gene diversity could be due to the repeat type and length together with method of analysis. In other words, when SSR loci containing dinucleotide repeats with a fragment size range of 130 to 200 bp were used, these fragments cannot be detected on agarose gel (Agrama & Tuinstra, 2003). To improve separation of alleles with small bp differences, capillary systems, acrylamide and metaphor gels are recommended (Agrama & Tuinstra, 2003; Yadav et al., 2007).

Acrylamide gels are reported to have high resolution power of separating nucleotide differences of one base pair, whereas 4 % metaphor gels can detect size differences (in bp) of ≈ 2 % (Senior et al., 1998).

This study identified two SSR loci (Xcump003 and Xcump012) with a PIC value of higher than 0.5 and the remaining loci were with lower PIC values. This may be resulted from the low number of SSR markers with di-nucleotide repeats used in this study. In general, SSRs with di-nucleotide repeats displayed a higher number of alleles than tri- and tetra-nucleotide repeats, and a direct relationship exists between PIC value and the number of repeat units (Ali et al., 2008). In this study, the analysed pearl millet genotypes showed unique alleles that were confirmed by bands found at different sizes of 100 bp DNA ladder. This observation supports the view that mutation induction through gamma irradiation technique caused genetic variations in the pearl millet varieties analysed in this study.

5.1.3 Cluster and genetic diversity analysis

At a similarity level of 51.8 %, the genotypes were clustered into two main clusters. This level of similarity was relatively low indicating a moderate level of genetic diversity among the putative mutant germplasm. This implies that the induced mutation managed to create putative mutant which are diverse at genetic level with lower similarity level compare to 68 % reported by McBenedict et al. (2016). Therefore, cluster analysis is a useful statistical tool to study the relationships among closely related accessions. Similarly, the Shannon-Wiener diversity value of this study is 2.59 which can be categorized as moderate in both species richness and evenness. Values of Shannon-Wiener diversity index above 3.5 in a given species are considered as indicators of high species

richness and diversity, and values below 1.5 are considered indicators of low species richness and evenness (Magurran, 1988).

According to Simpson's diversity index, 0 represents infinite diversity and 1 represents no diversity (Magurran, 1988). In this study, the Simpson's diversity index value of 0.93 was obtained suggestion low level of diversity among pearl millet genotypes. The results of various diversity measure used in this study indicated that the pearl millet genotypes revealed a moderate level of genetic diversity (51.8 % similarity index) and they are also moderate in both species richness and evenness. This may be justified by the fact that the mutants were generated from two parental lines and the gamma treatment didn't cause significant chromosomal rearrangements.

5.2 Biochemical characterization of pearl millet's putative mutant germplasm

5.2.1 Proximate composition analysis

Majority of proximate composition showed a significant difference ($P < 0.05$) within and among *Kangara* and *Okashana 2* derivatives and as well as in comparison to their parental lines. Crude protein content of the two parental lines was within the range reported by Kumaravel & Natarajan (2015). However, some of the pearl millet putative mutant showed exceptionally high crude protein content, though they were within a wider range reported by Deepak et al. (2012). Crude protein content of the putative mutants in this study showed significant increment of up to 24 % in comparison with parental lines, but the values were within the range reported by earlier studies (Devi, Vijayabharathi, Sathyabama, Malleshi, & Priyadarisini, 2014; Sade, 2009; Saleh et al., 2013). Protein content variations observed in this study could be due to both genotype (cause by induced mutation) and environmental variations (water availability, temperature and soil fertility)

during grain development (Omondi et al., 2012). In general, pearl millet is known for its higher protein content compare to other major cereal crops with more balanced amino acid profile and high protein efficiency ratio. Pearl millet is gluten-free and it has a therapeutic effect for those prone to gluten allergy and celiac disease (Rai, Gupta, Yadav, & Govindaraj, 2015; Saleh et al., 2013).

Ash content was within the range reported by Deepak et al. (2012), expect in the case of L10P18 and L5P11Rep2 mutants with values 4.25 and 5.16 %, respectively. Sinha (1980) cited by Kumaravel & Natarajan, (2015) reported the highest ash value of 6.90 % in pearl millet. There were significant difference ($p < 0.05$) observed in crude fibre content between parental lines and their corresponding mutant derivatives. Generally, a reduction of crude fibre content in mutant lines was observed as compared to the parental lines, and this could be resulted from other proximate composition incremental effect. All the crude fibre figures observed in this study were higher than the earlier studies (Devi et al., 2014; Nambiar, Dhaduk, Sareen, Shahu, & Desai, 2011; Saleh et al., 2013). However, the values were lower than range reported by Taylor (2004) cited by Issoufou et al. (2013) and Deepak et al. (2012).

Similarly, Abate and Gomez (1983/84) cited by Kumaravel & Natarajan (2015) reported a higher average crude fibre in pearl millet at 4.33 % that concurred with the majority of previous studies. In addition, the fibre fractions (NDF and ADF) content of pearl millet reported by Kumaravel & Natarajan (2015) were very low in comparison with the value obtained in this study. The NDF has been shown to be negatively correlated with dry matter intake, while ADF has been shown to be negatively correlated with digestibility of food stuffs (Palic, 2007).

In this study, it was observed that the two parental lines were richer in energy level than the putative mutants and such pattern was almost identical with fat and carbohydrates content. All putative mutants under this study, on the other hand, had less energy content in comparison with other reports (Deepak et al., 2012; Saleh et al., 2013; Sade 2009; Nambiar et al., 2011). However, higher fat content was observed among samples in this study than what is reported by Nambiar et al. (2011), Issoufou et al. (2013), Sade (2009) and Devi et al. (2011). Despite the values obtained in this study were high, they fall within the range reported by Deepak et al. (2012).

The observed carbohydrates contents were less than the values reported by Saleh et al. (2013) and higher than the values reported by Devi et al. (2011) but within the range reported by Deepak et al. (2012). The nutritional quality of pearl millet is as equivalent as that of maize but generally superior to sorghum in terms of protein content, quality, efficiency ratio (PER), and metabolisable energy levels (Saleh et al., 2013; Vadez, Hash, Bidinger, & Kholova, 2012).

5.2.2 Functional and anti-nutrients properties of pearl millet's putative germplasms

Functional properties have been defined as the intrinsic physico-chemical characteristics which affect the behaviour of protein in food system during processing, storage and preparation (Kiin-Kabari, Eke-Ejiofor, & Giami, 2015). Majority of functional and anti-nutrients properties showed a significant difference ($P < 0.05$) within and among *Kangara* and *Okashana 2* derivatives and as well as in comparison to their parental lines.

Majority of both *Kangara* and *Okashana 2* derivatives had higher water absorption capacity (WAC) than the 226 % reported by Sade (2009). However, three mutant lines

(L8P12, L5P11Rep2 and L10P13Rep1) derived from *Okashana 2* showed a significantly lower ($P < 0.05$) WAC values. Higher WAC values observed in the pearl millet seed flour could be due to the high protein content of the mutant lines , which has high affinity for water molecules (Ijarotimi et al., 2013).

Bulk density (g/cm^3) values found in this study were higher than the values reported by Gull et al. (2015). This variation could be due to the fact that bulk density is a measure of heaviness of the flour and generally affected by the particle size and the density of the flour (Gull et al., 2015). Bulk density is very important in determining the packaging requirement, material handling, energy density, texture and application in wet processing in the food industry (Gull et al., 2015; Ijarotimi et al., 2013).

Findings of the swelling power were in agreement with the early report by Ogori, Jatua, Apeh, & Adamu (2013). Using swelling power's classification indicators reported by Schoch & Maywald (1968), the pearl millet flour in this study found to be classified as highly restricted as it was below 16 g/g .

Starch swelling power is attributed to the strength and character of the micellar network within the starch granule and its hydration capacity because the determination is a weight measure of swollen starch granules and their occluded water (Bhupender, Rajneesh, & Baljeet, 2013; Ocloo, Bansa, Boatin, Adom, & Agbemavor, 2010). Therefore, as the temperature increased, the starch vibrated more vigorously, breaking intermolecular bonds and allowing hydrogen-bonding sites to engage more water molecules. The swelling behaviour of starch depends mainly on the amylose content, structure of amylose and amylopectin and presence of non-carbohydrate substances, especially in the presence of lipids acting as inhibitor of swelling (Tester and Morrison, 1990 cited by Bhupender et al. (2013)). Therefore, swelling power might be highly influenced by the higher lipid content

observed among samples in this study than what is reported earlier. Furthermore, the swelling power of flours depends on particle size, types of variety and processing methods or unit operations used (Chandra, 2013).

Pearl millets have polyphenols, tannin, phytic acid/phytate, goitrogens and oxalic acid as anti-nutrient components (Nambiar et al., 2011). Nutritional quality is considerably lowered by the presence of anti-nutritional factors leading to poor digestibility of proteins, carbohydrates and bioavailability of minerals (especially iron and zinc in cereal-based food for infants, young children and women in developing countries) (Sade, 2009; Suma & Urooj, 2014). Paradoxically though, these anti-nutrient found in pearl millet, have other benefits of anti-oxidant properties (Rai et al., 2015). Therefore, depend on their localization in cereal grains, the proportion of these anti-nutrients can be reduced by decortications, malting, germination, fermentation, roasting, flaking, and grinding (Sade, 2009; Saleh et al., 2013; Suma & Urooj, 2014). Those processes may also modify mineral bioavailability, improve their edible, nutritional, and sensory properties of a diet.

Phytic acid content of the unmalted pearl millet grain reported by Badau *et al*., 2005 cited by Suma & Urooj (2014) were less than the values obtained in this study. Therefore, the considerable amount of phytate contained in the pearl millet putative mutants in this study may have a negative influence on mineral bioaccessibility (Suma & Urooj, 2014).

Oxalate content observed in this study are within the range reported earlier (Suma & Urooj, 2014). Oxalate contents observed in this study is less than 50 mg/100 g, therefore they are classified as low oxalate foods (Suma & Urooj, 2014). Similar amounts of oxalates were widely found in many vegetables and fruits which do not pose a nutritional problem (Suma & Urooj, 2014). Consumption of high oxalate-containing foods may result in hyperoxaluria and subsequent formation of insoluble calcium oxalate (CaOx) and/or

magnesium oxalate (MgOx) crystals, a primary component of kidney stones (Al-Wahsh, Wu, & Liebman, 2012).

5.2.3 The minerals content of pearl millet's putative mutant germplasms

Majority of results showed significant difference ($P < 0.05$) within and among *Kangara* and *Okashana 2* derivatives and their parental lines was observed in mineral contents. Calcium content were below the range reported by Nambiar et al. (2011), Deepak et al. (2012); Sade (2009) and Saleh et al. (2013) expect for L5AP18 putative mutant which is within the stated range. However, apart from R9P5 putative mutant which was within the range reported as in Kumaravel & Natarajan (2015), the calcium contents were higher. Most potassium and phosphorus content obtained in this study were below the values reported by Deepak et al. (2012); Devi et al. (2014); Nambiar et al. (2011) and Sade (2009) and Deepak et al. (2012). Similarly, Magnesium contents detected in this study were below the range reported by Nambiar et al. (2011), Deepak et al. (2012) and Sade (2009). According to Deepak et al. (2012), high potassium and magnesium in pearl millet helps in lowering blood pressure and maintenance of cardiovascular health.

Generally, the grain minerals nutrient concentrations detected in this study were considerably wider in range and some of the values were below the range reported by the early studies. Such observed differences could be caused by the differences among the genotypes in absorbing different minerals from the soil and the ability to accumulate minerals in the grains (Velu, Rai, Sahrawat, & Sumalini, 2008). In addition, concentrations of minerals in grains are influenced by numerous complex and dynamic factors, including genotype, soil properties, environmental conditions and nutrient interactions (Bashir et al., 2014).

Greatest interest among microelements is the iron (Fe) and zinc (Zn) contents, for which widespread deficiencies with numerous adverse health effects have been reported worldwide, especially in populations of the developing countries heavily dependent of cereal-based diets (Rai et al., 2015). Iron content of the genotypes analysed in this study were within the range values reported by Nambiar et al. (2011). Other studies reported by Deepak et al. (2012); Sade (2009) and Saleh et al. (2013) also fall within the above range value. All the 14 genotypes tested showed more zinc content than the range value of 2.2 – 3.1 mg/1000 g reported by Nambiar et al. (2011), while the values in this study ranged from 3.4 – 6.9 mg/100 g.

5.3 Correlation analysis of SSR and biochemical markers of pearl millet putative mutants

Strong relationships among the grain biochemical markers reflected that either the genetic factors or the physiological functions for the uptake/translocation in the grain of these nutritional traits are interconnected. Results of the correlation analysis between SSR and biochemical markers are in line with Jiang et al. (2007) and Bashir et al. (2014) but below correlation coefficient ($r = 0.61$) reported by Abdellatif & Khidr (2010). Pearson's correlation analysis between SSR and biochemical markers of pearl millet putative mutants ranged between -0.44 - 0.70, suggesting that the two marker systems are not correlated (Mukaka, 2012). There is little if any correlation between biochemical markers (proximate composition, mineral nutrient contents, functional and anti-nutrients properties). In this study, low correlation was observed among the proximate composition biochemical markers, while moderate positive or negative correlations were observed among mineral contents and among functional and anti-nutrients properties biochemical markers. CH and energy, and SC and WAC revealed a significant high positive correlation while moisture

and OM, and energy and ash had a significant high negative correlation compare to classification reported by Mukaka (2012).

The lack of associations among the SSR and biochemical markers, as well between biochemical markers indicates that the application of single marker system may not be efficient in characterizing genotypes but can be used in combination. However, many previous reports have shown that grain yield was negatively associated with some of the mineral nutrient contents due to the dilution effect (Bashir et al., 2014; Jiang et al., 2007). These traits can be used as indirect selection criteria for yield.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

To the best of our knowledge, this is the first report on the genotypic and biochemical characterization of putative mutant pearl millets in Namibia. Generally, the study found that SSR markers are valuable for assessing genetic diversity in pearl millet putative mutant genotypes. In this study, genetic diversity was assessed based on the number of alleles/bands detected for each SSR loci. However, it could be better if the detected bands were sequenced to determine the extent of their distinctiveness and to have better estimation of genetic diversity and PIC. It is further recommended that more polymorphic pearl millet SSR marker could be used for efficient screening and profiling of the genotypes by saturating more regions of the pearl millet genome.

The results of biochemical markers (proximate analysis, nutritionally valuable minerals, anti-nutrients and functional properties of the seed flour) of pearl millet putative mutants showed significant variations. Therefore, there are significant difference ($P < 0.05$) within and among *Kangara* and *Okashana 2* derivatives and as well as in comparison to their parental lines probably caused by the mutation. Biochemical parameters of proximate content (moisture, ash, crude protein, fat, crude fibre and carbohydrates) were not consistent in up/down regulation pattern in comparison to their parental lines. Only total energy content (however direct correlated with crude protein, crude lipid and carbohydrate) showed a constant trend of down regulation between mutant derivatives and their parental lines.

R9P5, L8P5Rep2, L9P3Rep1, L10P18, L5P1Rep2 and L0P3Rep1 are selected for higher content of protein. However, L5AP18, L9P3Rep1 and L8P5Rep2 are selected for quality

trait such as WAC and swelling capacity. L5AP18 and R6P1-1 are selected for higher content of Fe and Zn minerals. It is known that seeds rich in minerals such as Zn can give agronomic advantages to the crop such as high seedling vigour, high level of disease resistance as well as high water-use efficiency of the crop (Bashir et al., 2014). The pearl millet genotypes analyzed in the present study displayed a wide range of variations in the studied biochemical traits and most of them are comparable to the contents reported in pearl millets cultivated worldwide.

The present study was able to characterised selected putative mutants of pearl millet lines using SSR markers and scored 108 different size bands that enabled the phenogram clustering of mutants based on genetic diversity. It further assessed variability using biochemical traits of selected putative mutant pearl millet lines, and identified significant variation among mutant lines and parental genotypes. Thus, assessment of genetic diversity in pearl millet germplasm and determination of their biochemical activities would help to know the breeding potential of each particular putative mutant.

Furthermore, due to positive correlations among different biochemical markers it is possible to develop high-yielding varieties that are rich in several deficient micronutrients. The lack of association between SSR and biochemical markers suggests the utilization of both markers systems in genetic diversity analysis. Therefore, it is recommended that both approaches i.e. genotypic and biochemical analyses are useful tools to study, register, maintain and improve pearl millet putative mutants in Namibia. This would eventually contribute to food security, value addition, conservation of genetic resources, protect the breeder’s rights and the availability of more data can be used further in marker assisted breeding schemes.

Finally, low yielding is one of the main challenges in country's pearl millet production, it is recommended to further study the simple sequence repeat (SSR) markers that can identify genomic regions linked to grain and stover-yield-related traits in these populations by association analysis. It is also recommended that conventional plant breeding should be run concurrent with biotechnology approach in order to obtain commendable results using both holistic approaches.

REFERENCES

Abdellatif, K. F., & Khidr, Y. A. (2010). Genetic diversity of new maize hybrids based on SSR markers as compared with other molecular and biochemical markers. *Journal of Crop Science and Biotechnology*, *13*(3), 139–145. http://doi.org/10.1007/s12892-010-0068-x

Agrama, H. A., & Tuinstra, M. R. (2003). Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. *African Journal of Biotechnology*, *2*(10), 334–340. Retrieved from http://www.academicjournals.org/AJB

Ahloowalia, B. S., Maluszynski, M., & Nichterlein, K. (2004). Global impact of mutation-derived varieties. *Euphytica*, *135*(2), 187–204. http://doi.org/10.1023/B:EUPH.0000014914.85465.4f

Al-Wahsh, I. A., Wu, Y., & Liebman, M. (2012). A comparison of two extraction methods for food oxalate assessment. *Journal of Food Research*, *1*(2), 233–239. http://doi.org/10.5539/jfr.v1n2p233

Ali, G. M., Yasumoto, S., & Katsuta, M. (2007). Assessment of genetic diversity in sesame (*Sesamum indicum L.*) detected by Amplified Fragment Length Polymorphism (AFLP) markers. *Electronic Journal of Biotechnology*, *10*(1), 0–0. http://doi.org/10.2225/vol10-issue1-fulltext-16

Ali, M. L., Rajewski, J. F., Baenziger, P. S., Gill, K. S., Eskridge, K. M., & Dweikat, I. (2008). Assessment of genetic diversity and relationship among a collection of US sweet sorghum germplasm by SSR markers. *Molecular Breeding*, *21*(4), 497–509. http://doi.org/10.1007/s11032-007-9149-z

Bado, S., Forster, B. P., Nielen, S., Ali, A. M., Lagoda, P. J. L., Till, B. J., & Laimer, M. (2015). Plant mutation breeding: Current progress and future assessment. In *Plant Breeding Reviews: 39* (pp. 23–88). Hoboken, New Jersey: John Wiley & Sons, Inc. http://doi.org/10.1002/9781119107743.ch02

Bashir, E. M. A., Ali, A. M., Ali, A. M., Melchinger, A. E., Parzies, H. K., & Haussmann, B. I. G. (2014). Characterization of Sudanese pearl millet germplasm for agro-morphological traits and grain nutritional values. *Plant Genetic Resources*, *12*(1), 35–49. http://doi.org/10.1017/S1479262113000233

Bhupender, S. K., Rajneesh, B., & Baljeet, S. Y. (2013). Physicochemical, functional, thermal and pasting properties of starches isolated from pearl millet cultivars. *International Food Research Journal*, *20*(4), 1555–1561.

Branzei, D., & Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. *Nature Reviews. Molecular Cell Biology*, *9*(4), 297–308. http://doi.org/10.1038/nrm2351

Bray, C. M., & West, C. E. (2005). DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytologist*, *168*(3), 511–528. http://doi.org/10.1111/j.1469-8137.2005.01548.x

Caballero, A., García-Pereira, M., Quesada, H., Vos, P., Hogers, R., Bleeker, M., … Fanara, J. (2013). Genomic distribution of AFLP markers relative to gene locations for different eukaryotic species. *BMC Genomics*, *14*(1), 528. http://doi.org/10.1186/1471-2164-14-528

Cardle, L., Ramsay, L., Milbourne, D., Macaulay, M., Marshall, D., & Waugh, R. (2000). Computational and experimental characterization of physically clustered simple

sequence repeats in plants. *Genetics*, *156*(2), 847–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11014830

Chandi, A., Milla-Lewis, S. R., Jordan, D. L., York, A. C., Burton, J. D., Zuleta, M. C., … Culpepper, A. S. (2013). Use of AFLP markers to assess genetic diversity in Palmer Amaranth (*Amaranthus palmeri*) populations from North Carolina and Georgia. *Weed Science*, *61*(136–145). http://doi.org/10.1614/WS-D-12-00053.1

Chandra-Shekara, A. C., Prasanna, B. M., Bhat, S. R., & Singh, B. B. (2007). Genetic diversity analysis of elite pearl millet inbred lines using RAPD and SSR markers. *Journal of Plant Biochemistry and Biotechnology*, *16*(1), 23–28. http://doi.org/10.1007/BF03321924

Chandra, S. (2013). Assessment of functional properties of different flours. *African Journal of Agricultural Research*, *8*(38), 4849–4852. http://doi.org/10.5897/AJAR2013.6905

Clarke, K. (2001). *Primer v5: user manual tutorial*. Plymouth UK: PRIMER-E.

Deepak, S., Niranjan-Raj, S., Mithöfer, A., & Shetty, S. H. (2012). Nutritional bio-fortification in pearl millet. *The European Journal of Plant Science and Biotechnology*, *6*(2), 87–90. Retrieved from http://pubman.mpdl.mpg.de/pubman/item/escidoc:1284556:4

Devi, P. B., Vijayabharathi, R., Sathyabama, S., Malleshi, N. G., & Priyadarisini, V. B. (2014). Health benefits of finger millet (*Eleusine coracana L.*) polyphenols and dietary fiber: a review. *Journal of Food Science and Technology*, *51*(6), 1021–40. http://doi.org/10.1007/s13197-011-0584-9

Dexheimer, T. S. (2013). DNA repair pathways and mechanisms. In *DNA Repair of Cancer Stem Cells* (pp. 19–32). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-007-4590-2_2

Dwivedi, S. L., Upadhyaya, H. D., Senthilvel, S., Hash, C. T., Fukunaga, K., Diao, X., … Prasad, M. (2012). *Millets: Genetic and genomic resources*. (J. Jannick, Ed.) (1st ed.). John Wiley & Sons, Inc. Retrieved from http://oar.icrisat.org/5375/1/Millets_genetic_and_genomic_resources_2012.pdf

El-Nahas, A. I., El-Shazly, H. H., Ahmed, S. M., & Omran, A. A. A. (2011). Molecular and biochemical markers in some lentil (*Lens culinaris Medik.*) genotypes. *Annals of Agricultural Sciences*, *56*(2), 105–112. http://doi.org/10.1016/j.aoas.2011.11.001

FAO/INFOODS. (2012). *FAO / INFOODS Guidelines for checking food composition data prior to the publication of a User / Table database -Version 1.0*. Rome. Retrieved from http://www.fao.org/fileadmin/templates/food_composition/documents/Guidelines_data_checking_final_oct2012.pdf

Fiehn, O. (2002). Metabolomics - The link between genotypes and phenotypes. *Plant Molecular Biology*, *48*(1–2), 155–171. http://doi.org/10.1023/A:1013713905833

Gill, S. S., Anjum, N. A., Gill, R., Jha, M., & Tuteja, N. (2015). DNA damage and repair in plants under ultraviolet and ionizing radiations. *The Scientific World Journal*, *2015*, 250158. http://doi.org/10.1155/2015/250158

Gulia, S. K., Singh, B., & Wilson, J. P. (2010). A simplified, cost-and time-effective procedure for genotyping pearl millet in resource-limited laboratories. *African Journal of Biotechnology*, *9*(20), 2851–2859. http://doi.org/10.5897/AJB09.150

Gull, A., Prasad, K., & Kumar, P. (2015). Physico-chemical, functional and antioxidant properties of millet flours. *Journal of Agricultural Engineering and Food Technology*, *2*(1), 73–75. Retrieved from http://www.academia.edu/11327988/Physico-chemical_Functional_and_Antioxidant_Properties_of_Millet_Flours

Guthridge, K. M., Dupal, M. P., Kölliker, R., Jones, E. S., Smith, K. F., & Forster, J. W. (2001). AFLP analysis of genetic diversity within and between populations of perennial ryegrass (*Lolium perenne L.*). *Euphytica*, *122*(1), 191–201. http://doi.org/10.1023/A:1012658315290

Horn, L. N., Ghebrehiwot, H. M., & Shimelis, H. A. (2016). Selection of novel cowpea genotypes derived through gamma irradiation. *Frontiers in Plant Science*, *7*(262), 1–13. http://doi.org/10.3389/fpls.2016.00262

Horn, L., & Shimelis, H. (2013). Scientific research and essays radio-sensitivity of selected cowpea (*Vigna unguiculata*) genotypes to varying gamma irradiation doses. *Academic Journals*, *8*(40), 1991–1997. http://doi.org/10.5897/SRE2013.5682

Horn, L., Shimelis, H., & Laing, M. (2015). Participatory appraisal of production constraints, preferred traits and farming system of cowpea in the northern Namibia: Implications for breeding. *Legume Research*, *38*(5), 691–700. http://doi.org/10.18805/lr.v38i5.5952

Horwitz, W., & Latimer, G. W. (2006). *Official methods of analysis of AOAC International*. AOAC International.

Hu, Z., Mbacké, B., Perumal, R., Guèye, M. C., Sy, O., Bouchet, S., … Miller, W. (2015). Population genomics of pearl millet (*Pennisetum glaucum (L.) R. Br.*): Comparative analysis of global accessions and Senegalese landraces. *BMC Genomics*, *16*(1), 1048.

http://doi.org/10.1186/s12864-015-2255-0

Ijarotimi, O. S., Adeoti, O. A., & Ariyo, O. (2013). Comparative study on nutrient composition, phytochemical, and functional characteristics of raw, germinated, and fermented Moringa oleifera seed flour. *Food Science & Nutrition*, *1*(6), 452–63. http://doi.org/10.1002/fsn3.70

Ipinge S.N.A. (1998). *A proposal for release of pearl millet varieties SDMV 93032 (Okashana 2) SDMV 92040 (Kangara)*. Windhoek, Namibia. Retrieved from http://www.the-eis.com/data/literature/Namibia National Sorghum and Pearl Millet Improvement Programme.pdf

Issoufou, A., Mahamadou, E. G., & Guo-Wei, L. (2013). Millets: Nutritional composition, some health benefits and processing — A review. *Emirates Journal of Food and Agriculture*, *25*(7), 501–508. http://doi.org/10.9755/ejfa.v25i7.12045

Jackson, S. P. (2002). Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, *23*(5), 687–696. http://doi.org/10.1093/carcin/23.5.687

Jiang, G.-L. (2013). Molecular markers and marker-assisted breeding in plants. In S. B. Andersen (Ed.), *Plant Breeding from Laboratories to Fields*. InTech. http://doi.org/10.5772/52583

Jiang, S. L., Wu, J. G., Feng, Y., Yang, X. E., & Shi, C. H. (2007). Correlation analysis of mineral element contents and quality traits in milled rice (*Oryza stavia L.*). *Journal of Agricultural and Food Chemistry*, *55*(23), 9608–9613. http://doi.org/10.1021/jf071785w

Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture. (2015).

Molecular characterization of mutant mermplasm: A manual. Seiberdorf. Retrieved from http://www-naweb.iaea.org/nafa/pbg/public/Molecular-Characterization-Mutant-Germplasm-Manual-2015.pdf

Jonah, P. M., Bello, L. L., Lucky, O., Midau, A., Moruppa, S. M., & Moruppa, Ω. S. M. (2011). Review : The importance of molecular markers in plant breeding programmes. *Global Journal of Science Frontier Research*, *11*(5), 5–12. Retrieved from https://globaljournals.org/GJSFR_Volume11/2-Review-The-Importance-of-Molecular.pdf

Kannan, B., Senapathy, S., Bhasker Raj, A. G., Chandra, S., Muthiah, A., Dhanapal, A. P., … Hash, C. T. (2014). Association analysis of SSR markers with phenology, grain, and stover-yield related traits in pearl millet (*Pennisetum glaucum (L.) R. Br.*). *The Scientific World Journal*, *2014*, 1–14. http://doi.org/10.1155/2014/562327

Kapila, R. K., Yadav, R. S., Plaha, P., Rai, K. N., Yadav, O. P., Hash, C. T., & Howarth, C. J. (2008). Genetic diversity among pearl millet maintainers using microsatellite markers. *Plant Breeding*, *127*(0), 33–37. http://doi.org/10.1111/j.1439-0523.2007.01433.x

Kenzhebayeva, S., Turasheva, S., Doktyrbay, G., Buerstmayr, H., Atabayeva, S., & Alybaeva, R. (2014). Screening of mutant wheat lines to resistance for fusarium head blight and using SSR markers for detecting DNA polymorphism. *IERI Procedia*, *8*, 66–76. http://doi.org/10.1016/j.ieri.2014.09.012

Kiin-Kabari, D. B., Eke-Ejiofor, J., & Giami, S. Y. (2015). Functional and pasting properties of wheat / plantain flours enriched with bambara groundnut protein concentrate. *International Journal of Food Science and Nutrition Engineering*, *5*(2),

75–81. http://doi.org/10.5923/j.food.20150502.01.

Kiprotich, F., Kimurto, P., Ombui, P., Towett, B., Jeptanui, L., Henry, O., & Lagat, N. (2015). Multivariate analysis of nutritional diversity of selected macro and micro nutrients in pearl millet (*Pennisetum glaucum*) varieties. *African Journal of Food Science*, *9*(3), 103–112. http://doi.org/10.5897/AJFS2014.1236

Kumar, M. B. A., Sherry, R. J., Varier, A., & Sharma, S. P. (2004). Seed esterases – A descriptor for characterization of pearl millet (*Pennisetum glaucum (L.) R.Br.*) genotypes. *Indian Journal of Genetics and Plant Breeding*, *64*(4), 267–270. Retrieved from www.researchgate.net/publication/234003663_Seed_esterases_-_A_descriptor_for_characterization_of_pearl_millet_Pennisetum_glaucum_L_RBr_genotypes

Kumar, S., Jacob, S. R., Tara Satyavathi, C., Dadlani, M., & Kumar, M. B. A. (2016). Utility of SSR markers in assessing the purity and identity of pearl millet hybrids. *Journal of Plant Biochemistry and Biotechnology*, *25*(1), 121–124. http://doi.org/10.1007/s13562-015-0329-3

Kumaravel, V., & Natarajan, A. (2015). Nutritive value of pearl millet grains for poultry feed: A review. *International Journal of Science, Environment and Technology*, *4*(1), 230–233. Retrieved from http://www.ijset.net/journal/537.pdf

Lemgharbi, M., Belhadi, B., Souilah, R., Terbag, L., Djabali, D., & Nadjemi, B. (2016). Biodiversity of pearl millet [*Pennisetum glaucum (L.) R. Br.*] in southern Algeria (Hoggar Region). *American Journal of Plant Sciences*, *7*(12), 1673–1684. http://doi.org/10.4236/ajps.2016.712158

Liang, H., Deng, Y., Wang, C., & Xu, X. (2016). A high-throughput DNA extraction

method from rice seeds. *Biotechnology & Biotechnological Equipment*, *30*(1), 32–35. http://doi.org/10.1080/13102818.2015.1088401

Lieber, M. R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annual Review of Biochemistry*, *79*, 181–211. http://doi.org/10.1146/annurev.biochem.052308.093131

Magurran, A. E. (1988). Diversity indices and species abundance models. In *Ecological Diversity and Its Measurement* (pp. 7–45). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-015-7358-0_2

Manivannan, A., Chhabra, A. K., & Nimbal, S. (2013). Characterization of pearl millet [*Pennisetum glaucum (L.) R. BR.*] genotypes by using superoxide dismutase (SOD) isozyme. *Indian Journal Of Agricultural Research*, *47*(6), 471–479. Retrieved from http://www.arccjournals.com/journal/indian-journal-of-agricultural-research/ARCC072

Manning, K., Pelling, R., Higham, T., Schwenniger, J.-L., & Fuller, D. Q. (2011). 4500-Year old domesticated pearl millet (*Pennisetum glaucum*) from the Tilemsi Valley, Mali: new insights into an alternative cereal domestication pathway. *Journal of Archaeological Science*, *38*(2), 312–322. http://doi.org/10.1016/j.jas.2010.09.007

Manova, V., & Gruszka, D. (2015). DNA damage and repair in plants - from models to crops. *Frontiers in Plant Science*, *6*, 885. http://doi.org/10.3389/fpls.2015.00885

Mathew, A. K., & Padmanaban, V. C. (2013). Metabolomics: The apogee of the omics trilogy. *International Journal of Pharmacy and Pharmaceutical Sciences*. http://doi.org/10.1038/nrm3314

Mba, C. (2013). Induced mutations unleash the potential plant genetic resources for food and agriculture. *Agronomy*, *3*, 200–231. http://doi.org/10.3390/agronomy3010200

Mba, C., Afza, R., Bado, S., & Jain, S. M. (2010). *Plant Cell Culture*. (M. R. Davey & P. Anthony, Eds.). Chichester, UK: John Wiley & Sons, Ltd. http://doi.org/10.1002/9780470686522

Mba, C., Guimaraes, E. P., & Ghosh, K. (2012). Re-orienting crop improvement for the changing climatic conditions of the 21st century. *Agriculture & Food Security*, *1*(1), 7. http://doi.org/10.1186/2048-7010-1-7

McBenedict, B., Chimwamurombe, P., Kwembeya, E., & Maggs-Kölling, G. (2016). Genetic diversity of Namibian *Pennisetum glaucum (L.) R. BR.* (Pearl millet) landraces analyzed by SSR and morphological markers. *The Scientific World Journal*, *2016*, 1–11. http://doi.org/10.1155/2016/1439739

Mukaka, M. M. (2012). Statistics corner: A guide to appropriate use of correlation coefficient in medical research. *Malawi Medical Journal*, *24*(3), 69–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23638278

Nakusera, F., Kadhikwa, G., & Mushendami, P. (2008). Unleashing the potential of the agricultural sector in Namibia. Retrieved from https://www.bon.com.na/Publications/Other-Publications/Occasional-Papers.aspx?page=2

Nambiar, V. S., Dhaduk, J. J., Sareen, N., Shahu, T., & Desai, R. (2011). Potential functional implications of pearl millet (*Pennisetum glaucum*) in health and disease. *Journal of Applied Pharmaceutical Science*, *1*(10), 62–67. Retrieved from http://r4d.dfid.gov.uk/Output/190751/Default.aspx

Neto, R. P. M., Pinho, E. V. R. Von, Carvalho, B. L., Pereira, G. S., & Neto, R. P. M. (2013). Identification of earl millet cultivars using both microsatellites and enzymatic markers. *Genetics and Molecular Research*, *12*(121), 1–14. http://doi.org/10.4238/2013.January.7.1

Nghishikungu-Horn, L. N. (2007). *Genetic diversity of sorghum (sorghum bicolor (l.) moench) and its wild relatives in Namibia using ssr and rapid analyses*. University of Namibia. Retrieved from http://hdl.handle.net/11070/356

Nweke, O., Osuji, C., Mairo, I. H., & Onyenekwe, P. C. (2014). A simple and rapid leaf genomic DNA extraction method for pearl millet (*Pennisetum glaucum L. R. Br*). *Journal of Applied Biotechnology*, *2*(2), 75. http://doi.org/10.5296/jab.v2i2.5440

Ocloo, F. C. K., Bansa, D., Boatin, R., Adom, T., & Agbemavor, W. S. (2010). Physico-chemical, functional and pasting characteristics of flour produced from Jackfruits (*Artocarpus heterophyllus*) seeds. *Agriculture and Biology Journal of North America*, *1*(5), 903–908. http://doi.org/10.5251/abjna.2010.1.5.903.908

Ogori, A. F., Jatua, M. K., Apeh, M. O., & Adamu, L. (2013). Chemical and functional characteristics of flours from blends of millet grain flour from distilled water soaking and malting (*Pennesitum glacum*). *International Research Journal of Food Science and Technology*, *1*(1), 1–7. Retrieved from http://acascipub.com/Journals.php

Oladosu, Y., Rafii, M. Y., Abdullah, N., Hussin, G., Ramli, A., Rahim, H. A., … Usman, M. (2016). Principle and application of plant mutagenesis in crop improvement: A review. *Biotechnology & Biotechnological Equipment*, *30*(1), 1–16. http://doi.org/10.1080/13102818.2015.1087333

Olasupo, F. O., Ilori, C. O., Forster, B. P., & Bado, S. (2016). Mutagenic Effects of

Gamma Radiation on Eight Accessions of Cowpea (*Vigna unguiculata [L.] Walp.*). *American Journal of Plant Sciences*, *7*(7), 339–351. http://doi.org/10.4236/ajps.2016.72034

Omondi, E. G. O., Makobe, M. N., Onyango, C. A., Matasyoh, L. G., Imbuga, M. O., & Kahangi, E. N. (2012). Nutritional evaluation of mutants and somaclonal variants of sorghum (sorghum. *Scientific Conference Proceedings*, *5*(L), 577–587. Retrieved from http://www.elearning.jkuat.ac.ke/

Parry, M. A. J., Madgwick, P. J., Bayon, C., Tearall, K., Hernandez-Lopez, A., Baudo, M., … Phillips, A. L. (2009). Mutation discovery for crop improvement. *Journal of Experimental Botany*, *60*(10), 2817–2825. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19516074

Pathirana, R. (2011). Plant mutation breeding in agriculture. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *6*(32), 1–20. http://doi.org/10.1079/PAVSNNR20116032

Pfeiffer, P., Goedecke, W., & Obe, G. (2000). Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. *Mutagenesis*, *15*(4), 289–302. http://doi.org/10.1093/mutage/15.4.289

Phillips, E. R., & McKinnon, P. J. (2007). DNA double-strand break repair and development. *Oncogene*, *26*(56), 7799–7808. http://doi.org/10.1038/sj.onc.1210877

Querci, M., Jermini, M., & Van den Eede, G. (2006). *Training course on the analysis of food samples for the presence of genetically modified organisms: User manual* (2006). Milan, Italy. Retrieved from http://mbg.jrc.ec.europa.eu/capacitybuilding/documentation.htm

Rai, K. N., Gupta, S. K., Yadav, O. P., & Govindaraj, M. (2015). Pearl millet improvement for food and nutritional security. Society for Millets Research, ICAR Indian Institute of Millets Research. Retrieved from http://oar.icrisat.org/8800/1/Chapter3.pdf

Ramakrishnan, M., Antony Ceasar, S., Duraipandiyan, V., Al-Dhabi, N. A., & Ignacimuthu, S. (2016). Assessment of genetic diversity, population structure and relationships in Indian and non-Indian genotypes of finger millet (*Eleusine coracana (L.) Gaertn*) using genomic SSR markers. *SpringerPlus*, *5*, 120. http://doi.org/10.1186/s40064-015-1626-y

Roessner, U., & Bowne, J. (2009). What is metabolomics all about? *BioTechniques*. http://doi.org/10.2144/000113133

Rohrbach, D. D., Lechner, W. R., Ipinge, S. A., & Monyo, E. S. (1999). Impact from investments in crop breeding: The Case of Okashana 1 in Namibia. International Crops Research Institute for the Semi-Arid Tropics. Retrieved from http://oar.icrisat.org/1160/1/RA_00391.pdf

Roychowdhury, R., & Tah, J. (2013). Mutagenesis — A potential approach for crop improvement. In *Crop Improvement* (1st ed., pp. 149–187). Boston, MA: Springer US. http://doi.org/10.1007/978-1-4614-7028-1_4

Sade, F. O. (2009). Proximate, antinutritional factors and functional properties of processed pearl millet (*Pennisetum glaucum*). *Journal of Food Technology Technology*, *7*(3), 92–97. Retrieved from http://www.medwelljournals.com/fulltext/?doi=jftech.2009.92.97

Saleh, A. S. M., Zhang, Q., Chen, J., & Shen, Q. (2013). Millet grains: Nutritional quality, processing, and potential health benefits. *Comprehensive Reviews in Food Science*

and Food Safety, *12*(3), 281–295. http://doi.org/10.1111/1541-4337.12012

Sapre, S. S., Singh, A., Saripalli, G. M., Patil, V. R., & Talati, J. G. (2014). Characterization of downy mildew resistant and susceptible genotypes of pearl millet (*Pennisetum glaucum L.*) using SDS-page and rapd markers. *Agricultural Science Digest* , *34*(3), 171–176. http://doi.org/10.5958/0976-0547.2014.00996.3

Schoch, T. J., & Maywald, E. C. (1968). Preparation and properties of various legume starches. *Cereal Chemistry*, *45*(6), 564–573. Retrieved from http://www.aaccnet.org/publications/cc/backissues/1968/Documents/chem45_564.pdf

Sehgal, D., Rajaram, V., Armstead, I. P., Vadez, V., Yadav, Y. P., Hash, C. T., & Yadav, R. S. (2012). Integration of gene-based markers in a pearl millet genetic map for identification of candidate genes underlying drought tolerance quantitative trait loci. *BMC Plant Biology*, *12*, 9. http://doi.org/10.1186/1471-2229-12-9

Senior, M. L., Murphy, J. P., Goodman, M. M., & Stuber, C. W. (1998). Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. *Crop Science*, *38*, 1088–1098. Retrieved from http://www.cropsci.ncsu.edu/maize/publications/Senior_etal.pdf

Shehata, A. I., Al-Ghethar, H. A., & Al-Homaidan, A. A. (2009). Application of simple sequence repeat (SSR) markers for molecular diversity and heterozygosity analysis in maize inbred lines. *Saudi Journal of Biological Sciences*, *16*(2), 57–62. http://doi.org/10.1016/j.sjbs.2009.10.001

Shu, Q. Y., Forster, B. P., & Nakagawa, H. (Eds.). (2012). *Plant mutation breeding and biotechnology*. Wallingford, UK: CABI. http://doi.org/10.1079/9781780640853.0000

Shu Q.Y. (Ed.). (2009). Induced plant mutations in the genomics era. Rome: Joint FAO/IAEA programme. Retrieved from http://www.fao.org/docrep/012/i0956e/I0956e.pdf

Sikora, P., Chawade, A., Larsson, M., Olsson, J., & Olsson, O. (2011). Mutagenesis as a tool in plant genetics, functional genomics, and breeding. *International Journal of Plant Genomics*, *2011*, 314829. http://doi.org/10.1155/2011/314829

Singh, K. S., Sujit, R., Swarup, R. C., & Dibyendu, N. S. (2010). DNA repair and recombination in higher plants: insights from comparative genomics of arabidopsis and rice. *BMC Genomics*, *11*, 443. http://doi.org/10.1186/1471-2164-11-443

Singru, R., Sivaramakrishnan, S., Thakur, R. P., Gupta, V. S., & Ranjekar, P. K. (2003). Detection of genetic variability in pearl millet downy mildew (*Sclerospora graminicola*) by AFLP. *Biochemical Genetics*, *41*(11–12), 361–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14994825

Stefanova, P., Taseva, M., Georgieva, T., Gotcheva, V., & Angelov, A. (2013). A modified CTAB method for DNA extraction from soybean and meat products. *Biotechnology & Biotechnological Equipment*, *27*(3), 3803–3810. http://doi.org/10.5504/BBEQ.2013.0026

Stich, B., Haussmann, B. I., Pasam, R., Bhosale, S., Hash, C. T., Melchinger, A. E., & Parzies, H. K. (2010). Patterns of molecular and phenotypic diversity in pearl millet [*Pennisetum glaucum (L.) R. Br.*] from West and Central Africa and their relation to geographical and environmental parameters. *BMC Plant Biology*, *10*, 216. http://doi.org/10.1186/1471-2229-10-216

Suma, P. F., & Urooj, A. (2014). Influence of germination on bioaccessible iron and

calcium in pearl millet (*Pennisetum typhoideum*). *Journal of Food Science and Technology*, *51*(5), 976–81. http://doi.org/10.1007/s13197-011-0585-8

Suprasanna, P., Mirajkar, S. J., & Bhagwat, S. G. (2015). Induced mutations and crop improvement. In *Plant Biology and Biotechnology* (Vol. 1, pp. 593–617). New Delhi: Springer India. http://doi.org/10.1007/978-81-322-2286-6_23

Tara Satyavathi, C., Tiwari, S., Bharadwaj, C., Rao, A. R., Bhat, J., & Singh, S. P. (2013). Genetic diversity analysis in a novel set of restorer lines of pearl millet [*Pennisetum glaucum (L.) R. Br*] using SSR markers. *Vegetos*, *26*(1), 72–82. http://doi.org/10.5958/j.2229-4473.26.1.011

Taylor, J. R. N., & Emmambux, M. N. (2008). 6 – Gluten-free foods and beverages from millets. In *Gluten-Free Cereal Products and Beverages* (p. 119–V). http://doi.org/10.1016/B978-012373739-7.50008-3

Tuteja, N., Singh, M. B., Misra, M. K., Bhalla, P. L., & Tuteja, R. (2001). Molecular mechanisms of DNA damage and repair: Progress in plants. *Critical Reviews in Biochemistry and Molecular Biology*, *36*(4), 337–397. http://doi.org/10.1080/20014091074219

Vadez, V., Hash, T., Bidinger, F. R., & Kholova, J. (2012). II.1.5 Phenotyping pearl millet for adaptation to drought. *Frontiers in Physiology*, *3*, 386. http://doi.org/10.3389/fphys.2012.00386

Varshney, R. K., Ribaut, J. M., Buckler, E. S., Tuberosa, R., Rafalski, A. J. & Langridge, P. (2012). Can genomics boost productivity of orphan crops? *Nature Biotechnology*, *30*, 1172–1176. http://doi.org/10.1038/nbt.2440

Velu, G., Rai, K., Sahrawat, K., & Sumalini, K. (2008). Variability for grain iron and zinc contents in pearl millet hybrids. *Jornal of SAT Agricultural Research*, *6*, 1–4. Retrieved from http://ejournal.icrisat.org/Volume6/Sorgum_Millet/KN_Rai.pdf

Wheeler, E. L., & Ferrel, R. E. (1971). A method for phytic acid determination in wheat and wheat fractions. Retrieved January 7, 2016, from http://www.aaccnet.org/publications/cc/backissues/1971/Documents/cc1971a39.html

Xu, Y. (2010). *Molecular Plant Breeding (Cabi)*. Oxfordshire, UK: Crop science society of America. http://doi.org/10.2135/cropsci2010.12.0004br

Yadav, O. P., Mitchell, S. E., Zamora, A., Fulton, T. M., & Kresovich, S. (2007). Development of new simple sequence repeat markers for pearl millet. . *Journal of SAT Agricultural Research*, *3*(1), 34–37. Retrieved from http://scholarcommons.sc.edu/cgi/viewcontent.cgi?article=1070&context=biol_facpub

Yadav, R. S., Sehgal, D., & Vadez, V. (2011). Using genetic mapping and genomics approaches in understanding and improving drought tolerance in pearl millet. *Journal of Experimental Botany*, *62*(2), 397–408. http://doi.org/10.1093/jxb/erq265

APPENDICE

APPENDIX A

A1: Ethical clearance certificate

STUDENT ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: FOS/53/2015 **Date: 17 August, 2015**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

Title of Project: Genotype and Biochemical Characterisation of Germplasm of Pearl Millet Mutant lines in Namibia Using Simple Sequence Repeat Markers
Nature/Level of Project: Masters

Principal Researcher: Filemon N. Shindume

Student Number : 200025201
Host Department & Faculty: Faculty of Science

Supervisors : Dr. G. Kahaka (Main) L. Horn (Co)

Take note of the following:
(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.
(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.
(c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.
(d) The UREC retains the right to:
  (i). withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
  (ii). request for an ethical compliance report at any point during the course of the research.

UREC wishes you the best in your research.

Prof. I. Mapaure
UNAM Research Coordinator
ON BEHALF OF UREC

A2: Research permission letter

University of Namibia, Private Bag 13301, Windhoek, Namibia
340 Mandume Ndemufayo Avenue, Pioneers Park
☎ +264 61 206 3111; URL.: http//www.unam.edu.na

Date: **30 November 2015**

TO WHOM IT MAY CONCERN

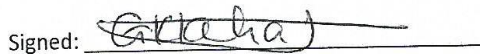
RE: RESEARCH PERMISSION LETTER

1. This letter serves to inform that student: **Filemon N. Shindume**, (Student number: **200025201**) is a registered student in the Department of Chemistry and Biochemistry at the University of Namibia. His/her research proposal was reviewed and successfully met the University of Namibia requirements.
2. The purpose of this letter is to kindly notify you that the student has been granted permission to carry out postgraduate studies research. The School of Post- graduate Studies has approved the research to be carried out by the student for purposes of fulfilling the requirements of the degree being pursued.
3. The proposal adheres to ethical principles.

Thank you so much in advance and many regards.

Yours truly,

Name of Main Supervisor: **Dr. G. Kahaka**

Signed: 

Dr. C. N.S. Shaimemanya

Signed:

Director: School of Postgraduate Studies

Tel: 2063523

E-mail: cshaimemanya@unam.na

APPENDIX B

Table B 1 List of pearl millet putative mutants that were used in this study.

It includes the Research Stations where they were collected from, sample size (g), generation and the gamma ray dose (Gy) level used for their physical mutation.

	Manheim Research Station							
	Kangara				**Okashana №2**			
Serial	**Variety Name**	**Gamma ray dose (Gy)**	**Generation**	**Sample weight (g)**	**Variety Name**	**Gamma ray dose (Gy)**	**Generation**	**Sample weight (g)**
1	L5AP18	400	M_6	128	L3P5	350	M_6	112
2	L7AP1A	400	M_6	114	L4P1	350	M_6	135
	Bagani Research Station							
3	R6P1-1	400	M_6	76	L8P12	350	M_6	94
4	R9P5	400	M_6	76	L10P18	3 50	M_6	92
	Omahenene Research Station							
7	L8P5Rep2	400	M_6	116	L5P11Rep2	350	M_6	168
8	L9P3Rep1	400	M_6	196	L10P13Rep1	350	M_6	168
9	*Kangara* (SDMV 92040)	0	-	5 000	*Okashana №2* (SDMV 93032)	0	-	5 000

Table B 2 Identification of the pearl millet samples as they appear in the agarose gels

Serial Number	Well identity
1	M = 100 bp DNA ladder
2	KFS (Kangara (SDMV 92040))
3	L5AP18
4	L7AP1A
5	R6P1-1
6	R9P5
7	L8P5Rep2
8	L9P3Rep1
9	OFS (Okashana №2 (SDMV 93032))
10	L3P5
11	L4P1
12	L8P12
13	L10P18
14	L5P11Rep2
15	L10P13Rep1
16	M = 100 bp DNA ladder

APPENDIX C

C1: DNA extraction protocol using the DNAExtractor (Clean) kit

1. Homogenize the sample mechanically, if necessary (use appropriate blender depending on the type of sample). For analysis of grains or whole meal homogenize at least 100 g of material.

2. Place 2 g of the (homogenized) sample into a 15 mL reaction tube, mix with 10 mL of the lysis buffer and add 10 µL proteinase K (for samples with coarse grain add 100 µL Proteinase K).

3. Incubate for at least 2 h at 60° C in an oven or waterbath under constant shaking or vortexing every 10 min.

4. Centrifuge for 5 min at >4,000x *g*.

5. Transfer 800 µL from the supernatant into a 1.5 mL reaction tube.

6. Optional: Add 5 µL of RNase. Incubate for at least 15 min at 60°C (Recommended for raw materials).

7. Add 600 µL chloroform and mix by vortexing.

8. Centrifuge for 10 min at >10,000x g.

9. Transfer 600 µL from the aqueous supernatant into a 1.5 mL tube. Add 2 µL glycogen solution and 480 µL 80% isopropanol and mix thoroughly. Incubate for 30 min at room temperature to precipitate the DNA.

10. Centrifuge for 10 min at >10,000x g to pellet DNA. Discard the supernatant.

11. Add 500 µL 75% ethanol to the DNA pellet and mix by vortexing.

12. Centrifuge for 5 min at >10,000x g. Discard the supernatant.

13. Centrifuge again for 1 min at >10,000x *g*. Carefully, remove all of the remaining supernatant using a micropipette and discard.

14. Dissolve the DNA pellet (may not always be visible) in 100 µL H2O or 0.1x TE buffer.

15. Optional, apply the whole DNA extract to a cleaning column.

C2: Column purification of extracted DNA using a sephacryl cleaning column

1) Re-suspend the resin in the column by vortexing. Open the bottom plug and loosen the cap one-fourth turn. Place the column in a 2.0 ml micro-centrifuge tube for support.

2) Pre-spin the column at 800 x *g* in a micro-centrifuge with a fixed-angle rotor for 1 min.

3) Place the column in a new 2.0 ml tube, remove and discard the cap and slowly apply the sample (10–100µL) to the top-centre of the resin, being careful not to disturb the bed.

4) Spin the column at 800 x *g* for 2 min. The purified sample is collected in the bottom of the support tube.

5) Take 5 µL of the DNA solution for the estimation of the DNA amount by gel electrophoresis or use nanodrop.

6) The 2.0 ml tubes with extracted DNA solution should be kept at +4 ºC until it is used.

C3: Protocol for gel preparation

1. Seal the edges of a clean, dry plastic gel-tray either with tape or other means. Position the appropriate comb so that complete wells are formed when the agarose solution is added.

2. Dilute 10x TBE buffer to prepare the appropriate amount of 0.5x TBE buffer to fill the electrophoresis tank and to prepare the gel or another suitable buffer i.e. 1x TAE buffer.

3. Weigh powdered agarose (for genomic DNA use a 1% gel and for PCR products use a 2% gel) and add it to an appropriate amount of 0.5x TBE buffer in an Erlenmeyer flask with a loose-fitting cap (usually 150 ml gel solution for a 15 x 15 cm gel-tray and 100 mL gel for a 15 x 10 cm gel-tray).

4. Heat the slurry in a microwave oven or in a boiling water bath until the agarose dissolves (check the volume of the solution after heating)

5. Cool the mixture to 50 - 60°C and add ethidium bromide (from a stock solution of 10 mg/ml) to a final concentration of 0.2 µg/mL and mix thoroughly.

6. Pour the solution into the gel-tray and allow the gel to set. The amount of gel used should correspond to a depth of approximately 3 - 5 mm.

7. After the gel is completely set, carefully remove the comb and the tape and place the gel in the electrophoresis tank. Add enough 0.5x TBE buffer to the electrophoresis unit to cover the gel to a depth of about 2 - 5 mm

C4: Protocol for gel electrophoresis

1. Load the DNA into the well. For extracted DNA mix 5 µL of the DNA mixed with 5 µL loading buffer and load 10 µL into the well. For PCR products add 10 µL of loading buffer to the PCR mixture (25 µL), mix and load 17 µL in the well. A DNA ladder should also be used to determine the size of the DNA. For genomic DNA use a 1% gel and for PCR products use a 2% gel.

2. Run the gel in 1x TAE buffer or another suitable buffer for approximately 15 to 40 min at 100 to 200 V.

3. The current is switched off and the gel is removed from the electrophoresis unit.

4. The gel is placed in staining solution for approximately 15 min with ethidium bromide (0.5 µg/mL in 1 x TAE) if ethidium bromide was not added during gel preparation

5. Optional: The gel can be destained for approx. 1 min in deionized H_2O.

6. The gel is documented using a UV transilluminator and a digital/polaroid camera. Care should be taken to wear protective glasses if the UV transilluminator is not enclosed.

APPENDIX D

D1: Proximate composition analysis protocols

D1.1 Moisture content

One gram of sample in pre-weighed crucible was placed in an oven (105 °C) for 24 h, cooled and reweighed according to AOAC 934.01 method (Horwitz & Latimer, 2006). The percentage moisture was calculated as follows:

$$\text{Moisture content (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where, W_1 - weight of the crucible, W_2 - weight of the crucible after drying at 105 °C and sample, and W_3 - weight of the crucible and the sample after cooling in airtight desiccators.

D1.2 Crude protein

Crude protein content was estimated using Leco CHN628 Determinator (Leco corporation, St. Joseph MI, USA) using the Dumas combustion method, which is a transformation of the sample to a gas phase by extremely rapid and complete flash combustion of a sample material (Palic, 2007). A weighed sample cup holder with foil is tarred and 0.1 g ± 0.05 g of sample added. All combustible materials in the sample are burned, water vapour and carbon dioxide are removed so the nitrogen-bearing combustion products include N^2 and various oxides of nitrogen pass through a reduction column filled with copper wires (600 °C) in which the nitrogen oxides reduced to nitrogen atoms. The clean sample gas (N^2) and a separate reference/carrier stream of helium (noble gas) pass over a detector and the visible peak was displayed. Percentage total crude protein for pearl millet's grains was calculated as:

$$\text{Total Crude protein (\%)} = \text{N (\%)} \times 6.25$$

D1.3 Crude fibre

Crude fibre was determined gravimetrically after chemical digestion and solubilisation of other compounds present (i.e. protein and starch) with diluted sulphuric acid and sodium hydroxide (Palic, 2007). A marked and weighed crucible containing 1 g ± 0.001 g of dry sample was attached to the Fiwe Raw Fiber Extractors extraction unit (Velp Scientifica, Milan, Italy) and first 150 mL of heated 1.25 % H_2SO_4 and 3 drops of n-Octanol (anti-foaming agent) was added to each tube and boiled/digested for 30 min, then the acid was drained and sample washed with hot distilled water.

Following the acid treatment, 150 mL of heated 1.25 % NaOH and 3 drops of n-octanol was added to each tube and boiled/digested for 30 min, then the base was drained and sample washed with hot distilled water. To remove traces of water, sample was washed 3 times with 20 mL of acetone. The crucible was removed and oven dried overnight at 105°C and allowed to cool down for 30 min in a desiccator, The samples were weighed (W_1). The Crucibles were transferred into a cool furnace and ashed at 550 °C in a muffle furnace RM4 – L5152 (Labcon, Transvaal, RSA) for 4 hours and reweighed after cooling (W_2). Percentage extracted fiber was calculated as:

Crube fibre (%) = $\frac{W_1 - W_2}{m} \times 100$, where W_1 - weight (g) of residue in crucible after drying, while W_2 - weight (g) of residue in crucible after ashing, and m - original sample weight (g).

D1.4 Neutral detergent fibre (NDF)

Neutral detergent fibre determines the insoluble portion of foodstuffs such as hemi cellulose, cellulose, lignin, cutin and silica (cell wall fraction). Extraction was done with neutral detergent solution and α-amylase under specific condition. During extraction, interfering metal ions are removed by compositing with EDTA and protein with sodium

lauryl sulphate, while other nitrogenous matter and non-available proteins are removed with sodium sulphate. The α-amylase converts starch to soluble sugar and 2-ethoxyethanol eliminates non-fibrous residues and serves as anti-foam.

NDF composition was determined according to Palic (2007) protocol. A marked and weighed sintered glass crucible containing 1 g ± 0.001 g of dry sample (W_1) was attached to the extraction unit of Fiwe Raw Fiber Extractors (Velp Scientifica, Milan, Italy) and 50 mL of cold neutral detergent solution (NDS) was added to each tube and boiled for 30 min. Then 2 mL α-amylase solution and additional 50 mL of cold NDS was added to the mix and boiled for 30 min. NDS was drained and sample washed with hot distilled water till traces of foam have disappeared. To remove traces of water, sample was washed with 20 mL of acetone three times. The crucible was removed and oven dried overnight at 105 °C. The samples were allowed to cool down for 30 min in a desiccator and weighed (W_2). The crucibles were transferred into a cool furnace and ashed at 500 °C in a muffle furnace RM4 – L5152 (Labcon, Transvaal, RSA) for 4 hours and reweighed after cooling (W_3). Percentage extracted NDF was calculated as:

NDF (%) = $\frac{W_2 - W_3}{W_1} \times 100$, where, W_2 - weight (g) of residue in crucible after drying, W_3 - weight (g) of residue in crucible after ashing, and W_1 - the original sample weight (g).

D1.5 Acid detergent fibre (ADF)

ADF procedure is a rapid method to determine lingo cellulose in grains, feeds, silages and other plant material. ADF measures cellulose, lignin, cutin and silica while NDF estimates hemi cellulose in foodstuffs. The samples were incubated with pepsin under acidic conditions for 4 hrs, then extracted with acid detergent solution and the remaining residue is dried and ashed.

A dried sample of 1 g ± 0.001 g was transferred into large glass tube (W_1). Pre-heat 50 mL pepsin solution at 45 °C - and added to each sample and incubated them for 24 hrs at 45 °C under constant shaking (Palic 2007).

Weighed sintered glass crucible was attached to the Fiwe Raw Fiber Extractors extraction unit (Velp Scientifica, Milan, Italy) and the incubated sample solution was quantitatively transferred to crucible and 100 mL of cold acid detergent solution (ADS) was added to each tube and boiled for 60 min at 75 °C. ADS solution was drained away and sample washed with hot distilled water. The samples were washed with 20 mL acetone three times to remove traces of water. The crucible was removed and oven dried overnight at 105 °C, then cooled down the sample for 30 min in a desiccator and weighed (W_2). The crucibles were transferred into a cool furnace and ashed at 500 °C in a muffle furnace RM4 – L5152 (Labcon, Transvaal, RSA) for 4 hrs and reweighed after cooling (W_3). Percentage extracted ADF was calculated as:

ADF (%) = $\frac{W_2 - W_3}{W_1} \times 100$, where, W_2 - weight (g) of residue in crucible after drying, W_3 - weight (g) of residue in crucible after ashing, and W_1 - original weight (g) of sample.

D1.6 Lipid/ fat

Lipid content was estimated using fat solvent Extractor 148 (Velp Scientifica, Milan, Italy) according to AOAC 920.39 (Horwitz & Latimer, 2006). A sample of 2.0 ± 0.001 g was mixed with 2.3 g anhydrous sulfate into a thimble and covered with absorbent cotton. A 2/3 of pre-weighed flask filled with petroleum ether (40–60 °C) was attached to thimble and the extraction unit. The solvent was evaporated from the cup to the condensing column. Extracted fat in the cup was then placed in an oven at 105 °C for 1 h and allowed to cool down and weighed. Percent of crude fat was calculated as:

Crube fat (%) = $\frac{MFR-MF}{m} \times 100$, where, MF - weight (g) of the flask, MFR - weight of flask with extracted residue (g), and *m* - the original sample weight (g).

D1.7 Ash and minerals

Ash and mineral contents were determined according to Association of Analytical Chemists (AOAC) numbers 923.03 and 984.27 (Horwitz & Latimer, 2006). Two grams of sample was added into a pre-weighed crucible was incinerated in muffle furnace RM4 – L5152 (Labcon, Transvaal, RSA) at 500 °C.

DM (%) = $\frac{W_2-W_3}{W_2-W_1} \times 100$, where, W_1 - the weight (g) of cleaned, dried, ignited, and cooled crucible, W_2 - weight (g) of the crucible and sample after incinerating at 500 °C, and W_3 - weight (g) of the crucible and sample after cooling in an airtight homogenized vessel.

D2: Mineral determination of pearl millet protocol

The AOAC 984.27 (Horwitz & Latimer, 2006) was used to determine the mineral compositions of the samples. Weighed 1 - 3 g of air dried sample and ashed at 550 ℃ overnight. Dissolve the sample with 5 cm^3 of HCl before evaporated to dryness. Then 5 cm^3 of HNO_3 was added in a hot water bath. The sample was filtered in 100 cm^3 volumetric flask and made up to the mark with deionised distilled water.

The filtered solution was loaded to an ICP iCAP 6000 Series Spectrophotometer (Thermo Scientific, Newington NH, USA). The standard curve for each mineral, such as calcium, potassium, sodium, phosphorus, magnesium, iron and zinc, was prepared from known standards and the mineral value of samples was estimated against that of the standard curve.

D3: Determination of anti-nutrient in pearl millet protocols

D3.1 Determination of phytic acid method

An indirect colorimetric method of Wheeler and Ferrel (1971) was used for phytate determinations. This estimation was made based on an iron to phosphorus ratio of 4:6. A sample of 5 g was extracted with 3 % trichloro acetic acid. The phytate was precipitated as ferric phytate and converted to ferric hydroxide and then soluble sodium phytate by adding sodium hydroxide. The precipitate was dissolved in 3.2 N hot HNO and the colour read immediately at 480 nm using UV/Vis 196 spectrometer (GBC Scientific Equipment, Braeside, Australia). The standard solution was prepared from ferric nitrate $Fe(NO_3)_3$ and the iron content was extrapolated from a $Fe(NO_3)_3$ standard curve. The phytate concentration was calculated from the iron results assuming a 4:6 iron: phosphorus molecular ratio.

D3.2 Method for determination of oxalate content

Oxalate was determined according to Horwitz & Latimer (2006) protocol. A 1 g of the homogenized sample was transferred into a 100 mL conical flask. Add 75 mL of H_2SO_4 (3 mol/L) to the flask. The solution was stirred intermittently with a magnetic stirrer for about 1 h and then filtered using Whatman No. 1 filter paper. The hot sample filtrated (80–90 °C) was collected and titrated against 0.1 N $KMnO_4$ solution to the point when a faint pink color appeared that persisted for at least 30 sec. The concentration of oxalate in each sample was estimated from the calculation: 1 mL 0.1 N permanganate = 0.006303 g oxalate.

D4: Methods for determination of functional properties of flour

D4.1 Water absorption capacity (WAC) protocol

Water absorption capacity of the homogenized samples were determined using method reported by Beuchat (1977) and cited by Ijarotimi et al (2013) with modification for small samples. Sample was weighed (2 g) and hydrated with 10 mL of distilled water at 25 °C for 1 h with manual stirring at 10 min intervals. The excess water was drained with a Whatman No. 2 filter paper with slight suction. The water absorption index was calculated as follows:

$$\text{WAC (\%)} = \frac{\text{Weight gained upon hydration}}{\text{Dry weight}} \times 100$$

D4.2 Bulk density protocol

Bulk density was determined using the method described by Okaka and Potter (1979) cited by Ijarotini et al. (2013). A 12.5 g flour sample was put into a 25 mL measuring cylinder. The cylinder was tapped continuously until a constant volume was obtained.

$$\text{Bulk density (g/cm}^3\text{)} = \frac{\text{Weight of flour (g)}}{\text{Flour volume (mL)}}$$

D4.3 Method for swelling power determination

Swelling power was determined using the method described by Leach et al. (1959) as cited by Ijarotini et al. (2013) with modification for a small sample. A 1 g flour sample was mixed with 10 mL distilled water in a centrifuge tube and heated at 80 °C for 30 min. The mix was shaken continuously during the heating period. The suspension was centrifuged at 1000 x *g* for 15 min. The supernatant was decanted and the weight of the paste was taken. The swelling power was calculated as:

$$\text{Swelling capacity (g/g)} = \frac{\text{Weight of precpitate /paste}}{\text{Weight of a dry flour}}$$