DIVERSITY AND GENOME-WIDE ASSOCIATION IN NAPIER GRASS (*Cenchrus purpureus* L.) COLLECTIONS FOR AGRONOMIC AND DROUGHT-TOLERANCE RELATED TRAITS

MSc. THESIS

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HAWASSA UNIVERSITY, HAWASSA, ETHIOPIA

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DIVERSITY AND GENOME-WIDE ASSOCIATION IN NAPIER GRASS (*Cenchrus purpureus* L.) COLLECTIONS FOR AGRONOMIC AND DROUGHT-TOLERANCE RELATED TRAITS

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ADVISORS' APPROVAL SHEET

This is to certify that the thesis entitled "**Diversity and Genome-Wide Association in Napier Grass (*Cenchrus purpureus* L.) Collections for Agronomic and Drought-Tolerance Related Traits**" submitted in partial fulfilment of the requirements for the degree of master with specialization in **Plant Biotechnology**, the Graduate Program of the Department/School of Plant and Horticultural Science, and has been carried out by **Hailu Lire Wachamo** Id. No SGS/PB/0014 under our supervision. Therefore, we recommend that the student has fulfilled the requirements and hence hereby can submit the thesis to the department

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DECLARATION

First, I declare that this research report was the actual result of my work and fully acknowledged all the materials used to support my research finding. I have submitted this MSc thesis for the partial fulfilment of the requirements of a Master of Sciences degree in Plant Biotechnology at Hawassa University and documented it in the university's library to be made available to borrowers for reference under the rules and regulations of the library. I declare that thesis has not been presented and submitted to any other institution anywhere for the award of an academic degree, diploma, or certificate. With these brief quotations, this MSc thesis is allowable without requiring special permission provided that an accurate acknowledgement of the source is made. Requests for permission for extended quotations from or reproduction of this MSc thesis in whole or in part may be granted by the Head of the College of Agriculture or the Head of the School of Graduate Studies of the Hawassa university college of Agriculture and ILRI when the intended use of the material is for scholarly interest. In all other instances, permission must be obtained from the authors.

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ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragmented Lengthy Polymorphism
BLINK	Bayesian-information and LD iteratively nested keyway
CSA	Central Statistical Agency
DNA	Deoxyribonucleic Acid
EIAR	Ethiopian Institute of Agricultural Research
EMBRAPA	Brazilian Agricultural Research Corporation
FAO	Food and Agricultural Organisation
FAOSTAT	Food and Agricultural Organization Statical Agency
FarmCPU	Fixed and random model Circulating Probability Unification
GAPIT	Genomic Association and Prediction Integrated Tool
GATK	Genome Analysis Toolkit
GBS	Genotyping by Sequencing
GWAS	Genome Wide Association Studies
ILRI	International Livestock Research Institute
MLM	Mixed Linear Model
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Randomly Amplified Polymorphic DNA
SNPs	Single Nucleotide Polymorphism's
SSA	Sub Saharan Africa
SSR	Simple Sequence Repeats
WGS	Whole Genome Sequencing

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Diversity and Genome-Wide Association in Napier Grass (Cenchrus purpureus L.) Collections for Agronomic and Drought-Tolerance Related Traits

Hailu Lire Wachamo, Temesgen Magule, Abel Teshome Gari, Chris Stephen Jones

ABSTRACT

Limited access to improved forages is one of the major factors affecting livestock performance in sub-Sahara Africa (SSA). Cenchrus purpureus **L** *is a C_4 perennial grass species native to SSA and it has attributes high yielding, resistance against most pests and diseases. However, it has received limited research attention and few genomic tools have been developed for it to date. Main aim of this study was for genetic diversity, identification of SNPs and genome wide association analysis on 109 core collections of Napier grass accessions from 16 countries. Results shows that more than a million SNPs were identified for analysis. Among sequenced 84 Napier grass accessions were phenotyped for two seasons, under different water stress conditions normal water condition, moderate water stress (MWS) and severe water stress (SWS). Agronomic traits such as plant height (PH), leaf width (LW) and length (LL), total fresh weight (TFW) and total dry weight (TDW) and nutritional traits such as Acid Detergent fibre (ADF), acid detergent lignin (ADL), Neutral detergent fibre (NDF), crude protein (CP), Metabolizable Energy (ME) and in-vitro organic matter digestibility (IVOMD) were measured. Significant differences for both agronomic and nutritional traits were observed and most traits showed higher mean value under MWS conditions. Furthermore, a genome-wide association study (GWAS) identified more than 100 SNPs, for both agronomic and nutritional traits, that were significantly associated ($P < 1.00E-05$) with traits of interest. The results obtained in the present study will enhance our understanding of complex agronomic and nutritional traits in Napier grass and these genomic tools will serve as a valuable resource in future breeding programs to select high yielding and drought-tolerant varieties of Napier grass, suited for different agroecological zones.*

Keywords: *Agronomic traits, Drought, GWAS, INDELs, Napier grass, Nutritional Trait, SNPs, WGS*

1. INTRODUCTION

Demand for animal products in developing worlds such as Africa is growing in response to the rapidly rising economy and urbanization (Kingston-Smith *et al.*, 2013; Rajendran *et al.*, 2022). Sub-Saharan Africa (SSA) comprises one-fourth of the global livestock population and 18% of the global bovine herd (Butterbach-Bahl *et al.*, 2020). However, annual milk and meat production remain low compared to the global average and, the livestock industry is yet to meet the increasing demand for animal products in the region (Balehegn *et al.*, 2021; FAO, 2021). One of the main reasons for the low productivity of the livestock industry is inadequate access to quality feeds and forages exacerbated recently by the risk of climate change (Balehegn *et al.*, 2020; Paul *et al.*, 2020). Common feeding sources (communal grazing lands) remain a major source of forages in SSA (Hanan and Kahiu, 2016); but, these sources are becoming scarce as a result of the inevitable population increase, climate change, and more land being allocated for food crops (Enahoro *et al.*, 2019).

Cropping systems (i.e. priority given for food crops than forages to ensure food self-sufficiency) and climatic risks (like drought) are constraining livestock feed supply and productivity in East Africa (Paul *et al.*, 2020). Though accessible feeds sources are not sufficient to feed the present livestock population and are available to a certain amount in the majority of cases during and after the rainy season (Hassanuur *et al.*, 2020). Hence, the small-scale livestock industry is under enormous pressure arising from declining feed resources due to climatic factors (Kumar and Roy, 2021), and rising prices for the available feeds and forages (Assefa *et al.*, 2012). Therefore, boosting feed resources through harnessing forage genetic resources is of paramount importance to contribute to the development of the livestock sector and therefore rural livelihoods and economic growth in SSA (Ates *et al.*, 2018; Juju *et al.*, 2020).

Several forage germplasm resources were collected at International Livestock Research Institute (ILRI) from 160 countries in collaboration with global partners; 43 % and 17% of those collections are from SSA and Ethiopia, respectively (Hanson *et al.*, 2020). Among this, a variety of annual and perennial grasses, legumes, trees and shrubs are traditionally grown by the farmers in SSA (Batello *et al.*, 2008), though less research attention was given to date (Mengistu *et al.*, 2017). Forage grasses such as Napier grass, *Urochloa brizantha*, desho grass (*Pennisetum pedicellatum*), buffelgrass (*Cenchrus ciliaris*) (Cantarutti *et al.*, 2021); herbaceous legumes (*Stylosanthes, Centrosema, Desmodium*, Lablab and *Macroptilium*) (Jimoh *et al.*, 2021); tree legumes (Acacia), silages (Alfalfa, oats) and crop residues (sorghum and corn); (Trees, natural vegetation, crop residues, and grazing are the most common feed resources in the region (Balehegn *et al.*, 2021).

Napier grass is amongst the most important tropical forage grasses native to SSA. It is cultivated as a multipurpose forage, primarily used to feed cattle in cut and carry feeding systems (Negawo *et al.*, 2017); because of its ability to withstand repeated cuttings and some degree of resilience against drought (Muyekho, 2015; Paudel *et al.*, 2018). Furthermore, it is the higher-yielding tropical grass species (Muyekho, 2015; Paudel *et al.*, 2018) and perennial availability under irrigated conditions (Haegele *et al.*, 2017; Muktar *et al.*, 2019). Easy establishment, fast-growing, and good palatability, when cut between six and eight weeks of regeneration, are some of the additional attributes of Napier grass (Archibald *et al.*, 2021; Habte *et al.*, 2020; Singh *et al.*, 2013).

Despite the aforementioned attributes and primary importance, particularly to small-scale farmers, Napier grass has received little attention from researchers to date (Negawo *et al.*, 2017). At present, farmer's varieties are threatened by novel biotic factors such as Smut (caused by *Ustilago kamerunensisis)* and stunt (caused by a *phytoplasma*), and abiotic factors like drought (Farrell *et al.*, 2002; Kariuki *et al.*, 2016; Sangsuwan and Dickinson,

2019). To limit the damage caused by biotic and abiotic threats, and to improve the nutritional value of farmer's varieties, breeding is a way forward for new varieties development program (Kingston-Smith *et al.*, 2013).

For the successful improvement of Napier grass, germplasms stocks persevered in genebanks', both in *ex-situ* and *in-situ*, are vital sources to initiate a breeding program (Pattanashetti *et al.*, 2015). Advanced initial knowledge on these resources is key for sustainable use of the available genetic resources and developing improved varieties (Peters *et al.*, 2021), thereby providing solutions for challenges that affect yield and other important agronomic traits like tolerance against diseases (such as smut and stunt) and climatic factors (like drought and frost) (Nassif and Tanji, 2017; Sandhu *et al.*, 2019; Singh et al., 2012). To date, a limited number of studies were carried out to understand the genetic diversity among germplasm collections of Napier grass (Hanson *et al.*, 2020). Molecular markers such as Simple sequence repeats (SSRs) (Negawo *et al.*, 2018); Amplified Fragment Length Polymorphism (AFLP) (Wanjala *et al.*, 2013); Randomly Amplified Polymorphic DNA (RAPD) (Okukenu *et al.*, 2020); Inter-Simple Sequence Repeat (ISSR) and Single nucleotide polymorphisms (SNPs,) (Wang *et al.*, 2020); were used for genetic diversity studies of Napier grass germplasm. Recently, genotyping by sequencing (GBS) was utilized to develop genome-wide markers for Napier grass and assess genetic diversity among accessions (Muktar *et al.*, 2019; Paudel *et al.*, 2018).

For appropriate germplasm conservation, use and further genetic improvement the aforementioned genomic tools are critical (Brummer and Wang, 2020; Muktar *et al.*, 2019). Though the GBS approach is significantly better approach than PCR based molecular marker on the other hand whole genome sequencing (WGS) is a method of choice due to the reduction in the cost of sequencing (He *et al.*, 2014). It gives a complete genomic DNA sequence of the particular organism and generates more accurate information that can rapidly

identify/select genes associated with specific characteristics and accelerate the conventional variety development processes with the support of bioinformatics tools (Yano *et al.*, 2016). Therefore, to accelerate the breeding programs on Napier grass, the WGS approach is advanced for the discovery of genome-wide markers suitable for marker-assisted selection and/or building genetic maps (Peace *et al.*, 2019). It also helps to quantify the genetic variability and hence efficient use of available germplasm and conservation strategies (Nakato *et al.*, 2021; Perez-De-Castro *et al.*, 2012).

The main aim of this study was focused on the phenotypic and genetic diversity of Napier grass accessions within the ILRI genebank. In addition, to carry out, a genome-wide association analysis (GWA), for traits of interest such as resilience against abiotic stress and nutritional quality aspects of selected genotypes. Thus, the tools developed in this study will enable forage breeders to apply advanced plant breeding procedures like genomic selection and marker-assisted breeding in their improvement. Specifically, the study aimed at:

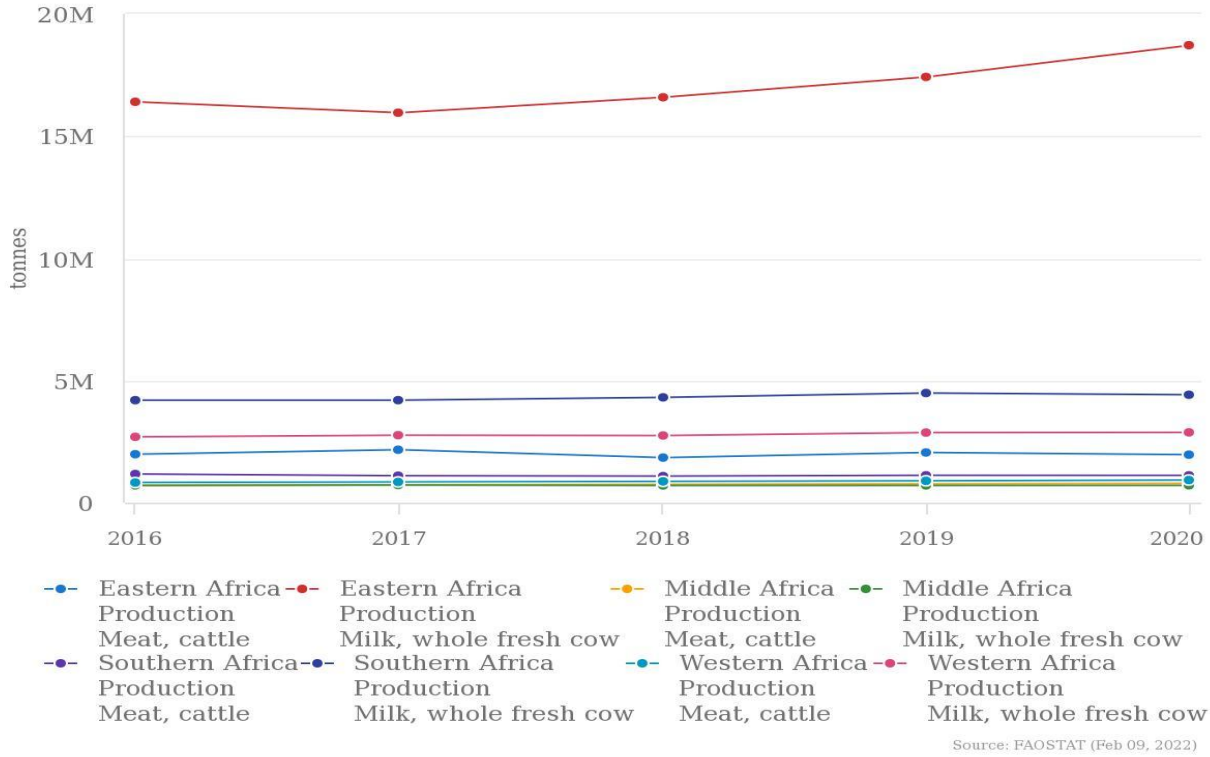
- Determining the extent of variability of Napier grass germplasm collection for agronomic and feed quality traits
- Describing genome-wide SNP variation across a global panel of Napier grass germplasm collections for a better understanding of the genetic structure of Napier grass
- Identifying genetic variants associated with agronomic and feed quality traits using Genome-Wide Association Study (GWAS)

2. LITERATURE REVIEW

2.1. Livestock Production and Productivity in Sub-Saharan Africa

In SSA, approximately one-third of rural people rely on livestock for a living (Gadekar, 2021; Ibeagha-Awemu *et al.*, 2019); and the region encompasses 18% of the global bovine herd, yet annual milk and meat production remains low compared to the global average (7000 tonnes) (Butterbach-Bahl *et al.*, 2020). Average meat and milk production in the SSA is below 2500 tonnes, which is more than half the global average, 7000 tonnes (FAOSTAT, 2021). While changing climatic conditions are global phenomena, their adverse effects are more severe on the livestock feeding systems in SSA, due to their dependency on rain-fed feeding schemes (Kabo-Bah *et al.*, 2021), resulting in a decline in food production of animal origin (i.e. meat and milk) (Patrick and Barkhuizen, 2020).

The main challenge affecting livestock production and productivity in the region is inadequate access to feeds and forages, which is available for a short period, mainly during the rainy season (Ayele *et al.*, 2021; McDermott *et al.*, 2010). Agriculture and livestock farming plays a vital role in the poor pastoral and agro-pastoral systems in SSA and its lack leads to continued economic decline and food security challenges (Birhanu *et al.*, 2021).



Source: (FAOSTAT, 2022)

Figure 1. Meat and Milk Production in Sub-Saharan Africa 2016-2020

2.2. Major Challenges of Livestock Production

2.2.1. Feed Quality and Supply

Livestock production and productivity in SSA are affected by various factors such as changing climate conditions like severe drought, flooding, land degradation, animal health and management practices (Ringler *et al*., 2010; Squires and Gaur, 2020). More importantly, limited access to quality forages and feed is the cause for the underperforming small-scale livestock industry in the region (Enahoro *et al.*, 2019; Mutimura *et al.*, 2015; Paul *et al.*, 2020). Therefore, to realize the full potential of livestock sectors in the region, a continuous supply of sufficient and quality feed is critical as any approach to boost production and productivity (Kriel, 2016). And also, a palatable feed source is an important aspect to

increase animal performance which is linked with feed quality like nutrient digestibility, chemical composition, and other attributes (Coleman and Moore, 2003).

2.2.2. Seasonal Feed Shortage

Various factors, such as biotic and abiotic stresses repeatedly affect the availability and quality of resources in SSA (Adugna, 2016; Lottering *et al.*, 2020). Among these factors, climate (delay in rainy season) has the greatest influence in reducing pasture quality and yield, disrupting forage seed production, and causing the appearance of biotic factors (diseases and pests) as well as direct effects on animal health, growth and reproduction (Adugna, 2016; Bakare *et al.*, 2020). Due to severe droughts, SSA potential vegetation is largely desert and semi-desert, shrub, and woodland, with only a small area of pure grassland resulting in the seasonal availability of feeds (Reid *et al.*, 2005). In addition, climate change-related challenges are expected to get worse in the future because additional other factors like continuous population increase, increasing energy demands, erratic weather conditions, shrinking arable land, and competition for water resources (Balehegn *et al.*, 2020; Diriba *et al.*, 2020).

2.3. Biodiversity of Feed and Forage Genetic Resources

Globally, around 12,000 species (650 to 785 genera) of grasses and 18 000 species of legumes are used as forage and fodder (Cherney and Cherney, 2011). As compared to the biodiversity of food crops available, genetic resources for feeds and forages lags far behind in terms of collection, characterization and genetic improvement (Priyadarshan and Jain, 2022). Hence, there is a need to increase the number of species and cultivars under collection, use, and preservation and recognize the work of end-users who preserve these genetic resources (Batello *et al.*, 2008).

Central, South America and Caribbean regions are the origin for genetically diverse legume forages (Kretschmer and Pitman, 2000) like Stylosanthes, Leucaena, Desmodium, Centrosema, and Gliricidia, while important grass genera, such as Urochloa (*syn. Brachiaria*), Pennisetum, Megathyrsus (*syn. Panicum*) and Digitaria are predominantly from SSA (Pengelly, 2015). Some important grass genera like Cenchrus and Bothriochloa have both African and Asian distributions (Sandhu et al., 2019). There are widely distributed grasses (Napier grass) and legumes (lablab) in SSA but genetic improvement and other breeding strategies and their adoption and use in the regions are limited and still underutilized (Barnes *et al*., 2021).

2.3.1. Feed and Forage Genetic Resources in SSA

There are various feed resources such as sown and /or planted grasses, herbaceous, dual-purpose legumes, shrub fodder legumes and trees which are among key components to improve livestock production and productivity (Casanova-Lugo *et al.*, 2022; Paul *et al.*, 2020); that can play important roles and achieve different goals in crop and animal production systems (Enahoro *et al.*, 2019). There are tropical and subtropical fodder resources, mainly legumes and grasses, which are used in the development of feeding systems for large and small scale animal production (Pengelly, 2015).

There are diverse germplasms of grain feeds (oats, corns) and forages (like local grasses, legumes, groomed pastures or woody forbs, and a wide variety of plants (Harris-Coble *et al*., 2021). But these resources are becoming scarce as a result of the inevitable population increase, more land being allocated for food crops, and changing climate affect conservation schemes (Balehegn *et al.*, 2021; Stavi *et al.*, 2021).

2.3.2. Feed and Forage Production Systems in Eastern Africa

Natural vegetation (natural grazing, crop residue, enset by-products (leaf and pseudostem), green feed (weeds and crop thinning), and sugarcane top) are the main forages in East Africa, mainly in Ethiopia (Dey *et al.*, 2021; Funte *et al.*, 2009); and also similarly, in Kenya; Uganda, Rwanda, and Sudan (except enset by-products (leaf and pseudostem), but its availability are mostly dependent on the rainy season and after harvesting time of crops (Paul *et al.*, 2020). Livestock production is the major component in the agriculture of Horn Africa; making it SSA's leader in milk production (contains 68% of Africa's milk production) (Bingi and Tondel, 2015). And its achievement depends on a better functioning of sufficient and quality feeding systems (Michael *et al.*, 2022), but a shortage of farmland, undulated topography, natural hazards, and absence of diversification in production are serious problems leading to poor performance of the livestock industry in the region (Paul *et al.*, 2020).

The livestock production system (particularly dairy production) is grouped as pastoral, agro-pastoral, the agro-pastoral in cooler and humid regions (crops and livestock) and sedentary schemes depending upon agro-climatic conditions, the purposes of production, available resources used, the extent of production, market orientation (Mengistu *et al.*, 2013). But the availability of sufficient and quality feeds and forages is very low which is threatened by seasonal climatic factors and diseases (Franzel *et al.*, 2014; Paul *et al.*, 2020). Loss of forage genetic resources (Hanson and Ellis, 2020); lack of improved high yielding and quality forage resources (Paul e*t al.*, 2020); more allocation of farmland for food crops, loss of biodiversity, severe drought, and other management practices are factors that limit forage production, particularly in eastern Africa (Lottering *et al.*, 2020; Wreford and Topp, 2020).

Furthermore, feed and feeding schemes of livestock are constrained by state restrictions on livestock mobility, grassland degradation, overgrazing, land tenure, land-use changes (Soumya *et al.*, 2022), the encroachment of invasive plant species, soil infertility, and inadequacy of grazing inputs and planting materials (Baumgard *et al.*, 2012; Ringler *et al.*, 2010). In addition to the above natural and man-made challenges, tropical forage research was given limited attention in the region leading to farmers using only landraces that are low yielding, susceptible to disease and pests and not amenable for mechanization (Balehegn *et al.*, 2021). As a result, there is an urgent need to focus and invest in enhancing tropical forages so that the underperforming livestock sector may reach its full potential (Kitalyi *et al.*, 2021).

2.3.3. Major Forage Resources, Production and Its Constraints in Ethiopia

Ethiopia has the largest livestock genetic resources and population in Africa and its main feed supplies are natural vegetation, crop residues, and grazing (CSA, 2016; Gebreyohanes *et al.*, 2021; Tolera *et al.*, 2012). Most commonly known forages in Ethiopia are natural pastures/ gross fodder (about 124 grass species; and 333 legumes species) and browse trees and root crop as well as roughages, agro-industrial by-products and concentrate compound feeds (Assefa *et al.*, 2012; Mengistu *et al.*, 2017; Tolera *et al.*, 2012). Natural pastures are the major fodder resources and represent 92.81% and 7% are other sources such as agricultural by-products (1.53%) and improved feeds and forages constitute only (0.31%) (Hassan *et al.*, 2020).

Hence, livestock productivity is profoundly dependent on natural sources of pasture in all parts of Ethiopia (Funte *et al.*, 2009; Kitaba and Tamir, 2007). However, it is not enough to meet the demands because it is limited by several factors such as ecological deterioration, drought due to climate change; unwanted weeds and bush invasion due to overgrazing; land

tenure due to investments; the decline in soil fertility due to soil erosion (Adugna, 2016; Guadu *et al.*, 2016; Mengistu *et al.*, 2017). Compound feeding, fodder, and forages are common feeding stuff worldwide which is also common in Ethiopia (Birhan and Adugna, 2014). These are harvested crop residues intended for animal feed are grown in a limited area for livestock that is the collection of legumes, grasses/herbs, maize, oats, alfalfa and other edible plants (Phelan *et al.*, 2015). Among key common forages, Napier grass is multipurpose high biomass yielding resource and known traditional grass grown in SSA and mostly Eastern Africa (Ethiopia, Kenya Uganda, Tanzania) (Umer and Nurusheva, 2020).

2.4. Overview of Napier Grass Description, Germplasms, Conservation and Breeding

2.4.1. Botany, Taxonomy, Distribution and Growing Ecology of Napier Grass

Napier grass (*Cenchrus purpureus* L.), is a multi-purpose forage(used as feed and forage, soil conservation, biofuel), native to SSA, used in intensive or semi-intensive agriculture (Mkhutche, 2020). It is known for its high biomass yield, adaptability under broader environmental conditions of growth (Muyekho, 2015; Negawo *et al.*, 2017); and is commonly grown in Ethiopia, Kenya, Uganda, Tanzania, Nigeria (Farrell *et al.*, 2002; Hassen, 2004; Mwendia *et al.*, 2006; Orodho, 2006). It is a perennial forage plant distributed and grown in the tropical and sub-tropical regions, known as a good source of palatable forage, at the early growth stage, and can rejuvenate after each harvest (Kamau, 2007; Knoll and Anderson, 2012; Singh et al., 2013). It is a monocotyledonous open-pollinated flowering plant that usually produces few full forms of seeds; so its main mode of propagation is by vegetative through stem cuttings (Dujardin and Hanna, 1985; Knoll and Anderson, 2012; Kustyorini *et al.*, 2019).

Genus *Cenchrus* has 140 known species, among which Napier grass is an important perennial C4 flowering cultivated species and its polyploidy level is an allotetraploid

(2n=4x=28, A'A'BB genome) (Yan *et al.*, 2021; Zhang *et al.*, 2020). It can yield 60-150 tons of green matter ha $^{-1}$ each year and is capable of withstanding repeated cuttings (four to six cuts per year), tolerates high temperatures, drought stress, low soil fertility, and other biotic stresses; but for its best growth temperature between 25-40 °C, and an altitude of above 2000m in the tropics (Dokbua *et al.*, 2020; Kamau, 2007; Rusdy, 2016; Yan *et al.*, 2020).

In addition, Napier grass is used as a biofuel source, for soil and water conservation, and as a trap crop in integrated pest management practices (Kabirizi *et al.*, 2015; Rengsirikul *et al.*, 2013). Once established in the main production field, it can grow and stay for a long time under good management practices (Hassen, 2004); and grow as a multi-cropping system that can be intercropped with legumes such as *desmodium*, *Macrotyloma* axillae, and *stylosanthes* (Knoll and Anderson, 2012; Rengsirikul *et al.*, 2013). Napier grass can grow in the wider types of soil but for better performance and high biomass yield, deep and fertile soil with good drainage is preferable (Nassif and Tanji, 2017).

2.4.2. Ecological Requirements and Management Practices of Napier Grass

Napier grass is a C4 grass it can grow at a wider altitude; for maximum yield, an altitude than 2000m is best and well ploughed and a fine planting field during establishment also favours establishment (Mengistu *et al.*, 2017). Depending upon growing environmental conditions and variety; appropriate nutrition, as well as irrigation supply, improve the performance and feeding quality of Napier grass (Mwendia *et al.*, 2018).

Napier grass is one of deeply-rooted, tall, fast-growing perennial grasses, and its main mode of propagation is by the cutting of stem and can withstand continuous harvesting, once established (Muyekho, 2015). Biomass yield and forage quality, as well as other nutritional attributes, are a function of variety, growing seasonal condition, growing environment and

agronomic and other management practices (like plant nutritional management; planting density, harvesting age, cutting height, water management, disease, and insect management practices) (Mukhtar *et al.*, 2003; Rusdy, 2016; Zewdu, 2008). More importantly significant difference in yield and nutritional attributes of Napier grass due to varieties reported which is important initial information for improvement programs for better forage quality and other agronomic traits (Wangchuk *et al.*, 2015); similarly growth and other attributes are influenced by genotype by environment interaction because of their difference in growth response to a specific environment (Kabirizi *et al.*, 2015).

2.4.3. Nutritional and Other Attributes of Napier grass

Poor nutritional quality is among the challenging factors affecting the production and productivity of livestock in SSA (Muia, 2000). According to Animasaun *et al.* (2018), Napier grass has lower forage quality than pearl millet. Furthermore, pearl millet has higher calcium, zinc, iron, and potassium whereas a higher percentage of acid and neutral detergent fibre and lower minerals were recorded for Napier grass (Wangchuk *et al.*, 2015; Zewdu, 2005). Nutritional and growth attributes of Napier grass are majorly controlled by growing altitude, agronomic management practices like harvesting time, and plant population (Mukhtar *et al.*, 2003), soil nutrient status and fertilizer application (Tessema *et al.*, 2011); other biotic factors such as diseases may also affect the nutritional content of Napier grass (Kitaba and Tamir, 2007; Rengsirikul *et al.*, 2013; Wangchuk *et al.*, 2015).

Table 1. Nutritional Summary of Napier Grass

Nutritional traits	Range (%)
Percentage Dry Matter Content	18-24
Ash	8.9-14.1
Crude Protein	6.4-12
Percentage Digestible Protein	55.7-62.2
Percent Neutral Indigestible Crude Protein	2.2-3
Percent Non-Fibre Carbohydrates	8.8-12
Percent Soluble Carbohydrate	3-5.8
Percent Acid Detergent fibre	47-52
Percent Neutral Detergent fibre	68-73
Percent Lignin Content	5.0-8.0
Percent Total Digestible Nutrients	46-50
Net Energy for Lactation (Mcal/Kg)	0.7-0.9
Net Energy for Growth (Mcal/Kg)	0.16-0.3
Net Energy for Metabolism (Mcal/Kg)	0.6-.0.8

Source: (Cuomo et al., 1996; Rusdy, 2016; Turano et al., 2016; Zewdu, 2005)

Besides, the nutritional quality of Napier grass is affected by the age of harvesting because the accumulation of required chemical composition is associated with the stage of harvesting (Takara and Khanal, 2015; Wangchuk *et al.*, 2015). For balanced livestock feeding and maximum yield, it is important to mix Napier grass with other forage sources for balanced mineral mixture because Napier grass was reported as deficient compared to a critical level in minerals elements (Table 2) and its nutritional content decreases as age increases (Aganga *et al.*, 2005).

Table 2. Mineral Composition of Napier Grass

Minerals	Concentration Mg/kg/DM	Minerals	Concentration Mg/kg/DM
Calcium	3.5	Zinc	50.4
Phosphorus	2	Manganese	33
Magnesium	1.7	Cobalt	2
Potassium	7.1	Iron	40.4
Copper	8		

Source (Aganga *et al.*, 2005)

2.4.4. Major Challenges of Napier Grass Production

Napier grass is well known for its high biomass yield when grown under irrigated conditions. But its yield and nutritional quality are constrained by various factors such as; drought (Gashaw *et al.*, 2014; Turano *et al.*, 2016); poor agronomic management practices (Mukhtar *et al.*, 2003), and/or biotic factors like smut and stunt diseases (Farrell *et al.*, 2002; Khan *et al.*, 2014). Some of these important factors that threat Napier grass are discussed below.

2.4.4.1. Abiotic and Biotic stresses

Napier grass is a drought-tolerant forage plant through changing its growing physiology in response to severe drought and water deficiency but its yield potential is affected as compared to normal growing conditions which are mainly from the direct effect of climate change like higher temperature and /or drought (Mwendia *et al.*, 2019; Wreford and Topp, 2020). In all growing altitudes, environmental stresses like drought, soil fertility, and poor agricultural practices significantly reduce the yield and quality of Napier grass (Mengistu *et al.*, 2017). According to Maleko *et al.* (2019) growth, biomass yield, and nutritional quality of Napier grass were affected by growing season and genotype interaction. To minimize

yield and quality loss arising from abiotic stresses developing improved varieties resilient against these stresses is the way forward (Habte *et al.*, 2020).

Biotic factors are one of the production constraints that affect the growth, and nutritional quality of forages including Napier grass (Farrell *et al.,* 2002; Khan *et al.,* 2014; Singh and Chahal, 2020). Insect pests (mites and nematodes), disease (viruses, fungal and bacterial) are among serious novel biotic factors that cause significant loss in yield and nutritional quality of Napier grass (Farrell *et al.,* 2002). Recently Smut (caused by *Ustilago kamerunensisis*) and stunt (caused by a *phytoplasma*) disease were reported as serious biotic factors affecting the productivity of the Napier grass in central and East Africa (Kenya, Tanzania, Uganda, Rwanda, Congo, and Cameron (Kawube *et al.*, 2014). Stunt disease causes complete yield loss (40-90%) and/ or even death of the plant (Wamalwa *et al.*, 2017). Similarly, yield loss due to smut is approximately 0.265 t ha^{-1} yr-1 (Mwendia *et al.*, 2007).

2.4.4.2. Lack of Improved Varieties and Poor Management Practices

The yield potential of Napier grass can be improved significantly with good agronomic management practices like fertilization, watering/irrigation, as well as insect pests and disease management (Maenetja, 2021). Napier grass is a promising forage resource but is yet to be fully domesticated and explored (Mwendia *et al.,* 2019; Paul *et al*., 2020; Simeão *et al.,* 2021; Turano *et al.,* 2016).

2.4.5. Progress of Napier Grass Germplasms Collection and Its Improvements

Forage germplasm collections are vital initial breeding tools that help to develop high-yielding and resilient varieties adaptable to wider climatic conditions and agroecology (Hanson and Ellis, 2020). Napier grass germplasm collections, characterization, and appropriate conservation are important strategies to enhance germplasm resources because every genetic improvement plan is mostly dependent on available germplasm and their initial

genetic variability (Habte *et al.*, 2020; Kawube *et al.*, 2015; Wanjala *et al.*, 2013). Besides, collection and appropriate maintenance is a fundamental approach against genetic erosion and rapid loss of germplasm from native biodiversity because of damage caused due to biotic, abiotic factors such as human interference, habitat destruction, air pollution and the invasiveness of non-native species, and deforestation (Anandhinatchiar *et al.*, 2020; Okukenu *et al.*, 2020)

The study of phenotypic and genotypic variability helps to identify desirable traits and enhance selective breeding for abiotic and biotic stresses, thus to achieve sustainable forage production, including in Napier grass (Lutatenekwa *et al.*, 2020; Wanjala *et al.*, 2013). Characterization based on morphological traits has long been used in conventional breeding and is now advanced by the use of molecular markers which speed up the process and permit optimal utilization of available diversity within a species and beyond (Anandhinatchiar *et al.*, 2020; Irshad, 2014). Napier grass germplasm collection, characterization, diversity study will contribute to a genetic improvement plan which helps to enhance improved varieties with good forage quality (Anandhinatchiar *et al.*, 2020).

2.4.5.1. Overview of Napier Grass Characterization and Genomic Assisted Breeding

2.4.5.1.1. Progress in Phenotypic Evaluation and Characterization of Napier Grass

Assessment of genetic variability among available germplasm helps further breeding programs by providing insight into polymorphisms that cannot be accounted for through phenotypic characterization (Anandhinatchiar *et al.*, 2020). Phenotypic characterization provides relevant morphological information that helps to identify some epigenetic information for those traits beyond genetics (Eichten *et al.*, 2014; McCouch *et al.*, 2012). Variability on biomass yields and nutritional content of Napier grass collections have been reported (Habte *et al.*, 2020; Maleko *et al.*, 2019; Turano *et al.*, 2016; Wouw *et al.*, 1999).

But phenotypic evaluations based on agro morphological traits cannot depict variability at the gene level and should be complemented by marker-assisted platforms (Muktar *et al.*, 2019; Pattanashetti *et al.*, 2015).

2.4.5.1.2. Genomic Selection, Characterization, and Breeding of Napier Grass

Napier grass has a long vegetative phase which makes it difficult to identify its germplasm based on only its agro-morphological traits (Bhandari *et al.*, 2006). Thus, evaluating genetic diversity with the help of molecular markers offers more accurate, fast, non-expensive technology that complements phenotyping, to identify relationships and purity among germplasm collections, populations, and species (Anandhinatchiar *et al.*, 2020; Kawube *et al.*, 2015; Muktar *et al.*, 2019).

DNA-based markers are among the essential tools for diversity study and breeding with a variety of applications including genome mapping, gene tagging, genetic diversity, and phylogenetic analysis (Irshad, 2014; Ortiz, 2002). There are various molecular markers like non-PCR-based (RFLP) and PCR-based markers (RAPD, AFLP, SSR, SNP); used for genetic diversity study of forages including Napier grass (Kandel *et al.*, 2016). Since sequencing costs became gradually lower, Single Nucleotide Polymorphism (SNPs), have gained high popularity due to their genome-wide coverage, even though it is only a bi-allelic type of marker (Wang *et al.*, 2020).

2.4.5.1.3. GBS and GWA Analysis of Napier Grass

Applying genomics to forage improvement programs is vital to accelerate conventional breeding by targeting key genes behind traits of interest and such tools are already in use in temperate forage like ryegrass (Genus Lolium) (Brummer and Wang, 2020; Habte *et al.*, 2020; Mishra and Singh, 2020). Developing and applying genomic tools contribute towards fast-tracking breeding efforts in Napier grass which has a perennial nature and is difficult to

improve through the conventional breeding approach (Ahmar *et al.*, 2020; Mishra and Singh, 2015). A whole-genome sequencing approach is a new tool that supports a genetic improvement plan through the formation of suitable reference genomes and their wild relatives to implement novel methodologies such as genomic selection (GS), genome-wide association studies (GWAS), epigenomics, and genome editing (Schreiber *et al.*, 2018).

A marker-trait association study was done in Napier grass (Habte *et al.*, 2020); the recent report indicated that diversity study and construction of high-density genetic mapping was done by sequencing which is one of the new insights to diversity study of Napier grass (Muktar *et al.*, 2019; Paudel *et al.*, 2018). However, despite this progress more genomic tools are needed for advanced improvement plans of Napier grass and other animal forage plants (Nuccio *et al.*, 2018; Paudel *et al.*, 2018). Also, more studies on the GWAS to identify important agronomic traits for further breeding; identification of molecular markers for diversity study in available germplasm collections is of paramount importance to address the necessary genomic study of Napier grasses (Azevedo *et al.*, 2012; Habte *et al.*, 2020; Kandel *et al.*, 2016).

Genome-wide association studies are one of the approaches for identifying the genomic regions responsible for the important agronomic traits like resistance to drought, high yielding, resistance to common diseases and other related qualitative and quantitative traits (Hirschhorn and Daly, 2005; Wang *et al.*, 2020). Identification of QTLs and/or molecular markers nearby the gene of interest, associated with important agronomic traits, facilitate the transfer of those traits into target populations via conventional approaches or through a genetic transformation which is a robust tool to make changes at a distinct locus in the genome, even at the individual nucleotide level (Brummer and Wang, 2020; Chai and Wang, 2020). Moreover, GWAS combines a wide-ranging and unbiased investigation of the genome with the power to detect common alleles in different loci with modest phenotypic

effects and hence it is also a powerful approach for dissecting complex traits (Akiyama, 2020; Grenn *et al.*, 2020; Wang *et al.*, 2020). Genomic tools fasten breeding effeort through clearly complementing conventional approach.

2.4.5.1.4. Prospects of Napier Grass Towards Full Domestication

There is progress on Napier grass improvement like germplasm collection, characterization, evaluation, and selection as well as a genomic study (Anandhinatchiar *et al.*, 2020; Muktar *et al.*, 2019; Nassif and Tanji, 2007). The main improvement objectives of Napier grass are developing resistant and/or tolerant varieties against smut and stunt diseases, and increasing forage quality such as crude protein content (Anandhinatchiar *et al.*, 2020; Kingston-Smith *et al.*, 2013; Mukhtar *et al.*, 2003). Limited genomic tools are available to date for Napier grass with the first reference genome published (Yan *et al.*, 2021) and with only two GBS studies to date (Muktar *et al.*, 2019; Paudel *et al.*, 2018). This is mainly because of lack of awareness, little attention of policymakers, lack of cheap and quality forage seed, and poor market linkages for inputs and outputs (Ndah T *et al.*, 2017; Sejian *et al.*, 2021). This grass is a key perennial traditional forage in SSA, with limited genetic resources for its improvement (Muktar *et al.*, 2021). Therefore, developing more genomic tools offers opportunities to apply modern breeding tools such as marker-assisted selection (MAS) and genomic selection (GS) to complement the traditional breeding approach (Simeão *et al.*, 2021).

3. MATERIALS AND METHODS

3.1. Plant Materials and Experimental Site

Among 109 Napier grass accessions collected and conserved at ILRI genebank and used in this study (Table 3 and Appendix Table 1), eighty-four accessions were phenotypically evaluated at Bishoftu as described by Muktar *et al.*, (2019), the site located 48 km southeast of Addis Ababa East Shewa Zone, Oromia Region). The field trial site is geographically located at $8^0$47"20' N $38^0$59"20' E, altitude 1800 (masl), annual rainfall (875mm), soil type alfisol maximum, average, and minimum Temperature (^{0}C) of 25,19, and 11 respectively. The trial was established in August 2017 and data collection was carried out between 2018-2020 as previously described by (Muktar *et al.*, 2019). In addition, all the 84 phenotyped accessions in the field trial (Figure 2) and an additional 24 that were not phenotyped were genotyped at the WGS level through Illumina sequencing tool (Table 3).

Figure 2. Field phenotyping of Napier grass accessions at Bishoftu

3.2. Field Trial and Experimental Design

Eighty-four Napier grass accessions were arranged in a partially replicated (p-rep) design and replicated four times for phenotyping as previously described by Muktar *et al.* (2019). Six stem cuttings from respective accessions were planted in a single row allowing 750 mm spacing between plants and rows. After six months of the establishment, a standard cut of 50 mm above ground was carried out before drought stress conditions were imposed at the beginning of 2018. During the dry season (DS), two blocks were irrigated to a volumetric soil water content (VWC) of approximately 20% (now onwards called moderate water stress (MWS) and the other two blocks were irrigated with a reduced amount of soil moisture, which corresponds to a VWC of about 10% (now onwards called severe water stress (SWS). Drip irrigation was paused during the wet season (WS) and VWC for all the blocks was approximately 30%. Soil moisture content was checked by using a Delta soil moisture probe (HD, England). Overall, 12 harvests were conducted, following every eight weeks of regrowth, in both wet and dry seasons. Phenotypic scores such as agronomic performance and feed quality traits of Napier grass accessions were collected under both moderate and severe water stress moisture conditions.

3.3. Agronomic and Nutritional Data Collected

A total of six growth and forage biomass yield traits, like plant height (PH) (cm), leaf length (LL) (mm), leaf width (LW) (mm), tiller number (TN), average total fresh weight per plant (TFW) (g) were measured after every eighth week of each harvest from six randomly selected plants, per accession, in each treatment condition. In addition, total dry weight per plant (TDW) (g) after oven drying 600gram fresh weight at 65 °C for 72 hrs was recorded at every harvest. Three hundred grams of the whole plant was oven-dried for nutritional trait analysis samples were ground into a powder fine enough to pass through a 1 mm sieve and

scanned using Near-Infrared Spectroscopy (NIRS) (FOSS Forage Analyzer 5000 with software package WinISI II) to estimate feed quality traits.

Seven nutritional traits like acid detergent fibre (ADF) (%), acid detergent lignin (ADL) (%), crude protein (CP) (%), Dry matter (DM) (%), *in vitro* organic matter digestibility (IOMD) (%), metabolizable energy (ME) (%), neutral detergent fibre (NDF) (%), organic matter (OM) (%) were measured by following procedures described by (Choudhary *et al.*, 2009).

3.4. Genotyping

3.4.1. DNA Extraction and Quality Control

Young leaf tissue was collected from respective 109 accessions (Table 3 and Appendix Table 1) and subjected for isolation of genomic DNA using the procedure as described by Qiagen DNeasy® Plant Mini kit (250) (Qiagen Inc., Valencia, CA) method. The DNA quality and quantification; was checked using a spectrophotometer and agarose gel electrophoresis. Before library preparation, DNA quality was checked on 1% agarose gels and DNA purity was checked using the Nanophotometer® spectrophotometer (IMPLEN, CA, The USA); and DNA concentration was measured using the Qubit® DNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). High-quality DNA with a minimum of 50 ng/µl was used for Illumina whole-genome sequencing at a depth of 20x.

3.4.2. Library Preparation and Sequencing

The construction of the sequencing library was created using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, England) and following manufacturers' recommendations. The 1µg genomic DNA was randomly fragmented to a size of 350bp by Bioruptor, then DNA fragments were narrowly size selected with sample purification beads. The selected fragments were then polished at the end, A-tailed, and bound with the full-length adaptor. After these treatments, these fragments are filtered with beads again. Finally,

the library was analysed for size distribution by Agilent2100 Bioanalyzer and quantified with real-time PCR. Libraries were sequenced by Illumina high-throughput sequencer with a paired-end sequencing strategy. Following library optimization and preparation, DNA sequencing was performed by the Novaseq platform and end readings of 150 bp were generated. Library preparation and sequencing was conducted by Novogene (https://en.novogene.com).

3.4.3. Genome-Wide SNP Discovery

Once raw sequence reads were received, the quality of reads was checked by the MultiQc tool (Ewels *et al.*, 2016). Afterwards, raw reads were trimmed and filtered by a trimmomatic tool (Bolger *et al.*, 2014) to remove remnant adaptor sequences and get rid of low quality reads ahead of the mapping. Cleaned reads were mapped to Napier grass reference genome with Burrows Wheller Aligner (BWA) which is a software package for mapping low-divergent sequences (Li and Durbin, 2009). Once bam files were generated, from the previous step, variant calling was carried out by Genome Analysis Toolkit (GATK3.8) (McKenna *et al.*, 2010). GATK generated a vcf file which was filtered by BCftools/1.8 (Li *et al.*, 2009). The SNP filtering only kept SNPs that are biallelic, polymorphic, read depth above 10 and below 300, mapping quality (GQ>20) and minor allele frequency above 0.85.

3.5. Agronomic and Nutritional Data Analysis

All the collected agronomic and nutritional traits were used for the analysis by using R-software version 4.0.2 for variance analysis in the library Agricolae (de Mendiburu and de Mendiburu, 2019). Pearson Correlation for an inter-trait association for both agronomic and nutritional trait analysis by using corr package in the R-Software. For cluster and principal coordinate analysis, the optimum cluster number and membership for respective accessions of Napier grass was done using FactomineR r-package for the analysis of the contribution of

nutritional and agronomic traits and to visualize the cluster plot fviz_cluster function of the R package factoextra. was used (Kassambara *et al.,* 2017).

3.6. Genomic Data Analysis and

3.6.1. Cluster and STRUCTURE Analysis

By using filtered SNPs STRUCTURE analysis was carried out and admixture-based clustering was used in structure V 2.3.2. and run ten independent times for each K value ranging from 1 to 10 with a burn-in of 100,000 iterations and 50,000 iterations for the analysis. The inference of true K, using an ad-hoc statistic ΔK, was determined based on the second-order rate of change in the log probability of data between consecutive values. The generated results were processed using Structure Harvester's web-based version ***https://taylor.biology.ucla.edu/StructureHarvester/***).

3.6.2. Phylogenetic Relationship and Principal Coordinate Analysis

Phylogenetic trees were constructed with filtered and high-quality SNPs and both the unweighted neighbour-joining method and the hierarchical clustering method based on the dissimilarity matrix was calculated with Manhattan index and visualized using R-software packages in a library (Ape, cluster) Version 4.0.2. A neighbour-joining tree based on a simple matching dissimilarity coefficient was constructed.

3.6.3. Genome-Wide Study (GWAS) Analysis

Mean agronomic and nutritional values were used for marker-trait association analysis. A marker-trait association was performed for each trait separately with multi-locus GWAS algorithms Fixed and random model Circulating Probability Unification (FarmCPU) (Lipka *et al.,* 2012) and Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK) Models (Huang *et al.*, 2019) implemented in GAPIT software package

within the R environment (R core team 2021). Missing data in the genotypic matrix were imputed by Beagle (Browning *et al.*, 2018). The population structure was accounted for by including two principal components in the subsequent analysis of the data. The distribution of observed vs. expected −log10(p) values were visualized using Quantile–Quantile (Q–Q) plots to test the fitness of GWAS models for both agronomic and nutritional traits (Sharma *et al.*, 2018); significant marker-trait associations, corresponding to putative QTLs, were determined by the P-value. Significantly associated SNPs (-Log10(P-value)> 5.0) were annotated against gramene plant database (https://www.gramene.org/) and NCBI database to check if the region containing these SNPs play a similar role in other grass species.

4. RESULTS

4.1. Variability of Agronomic and Nutritional Traits of Napier Grass Accessions under Different Soil Moisture Conditions

4.1.1. Agronomic and Nutritional Variability

For all the agronomic traits significantly *($p<0.05$*) different responses were recorded between accessions over growing moisture conditions (Table 4 and Appendix Table 2). Except for LW and TN, the rest of the agronomic traits were significantly affected by moisture conditions. Furthermore, traits like PH, TFW and TDW were significantly affected by the interactive effect of accessions and season. Higher values in agronomic traits were recorded during the wet season versus the dry season. A similar trend was also shown during the dry season, traits such as PH, TFW and TDW were higher in MWS vs SWS conditions (Figure 3A, 3B, 3C and 3D).

Similarly, there was a significant difference *($p<0.05$*) for feed quality traits between accessions under different growing seasons, and moisture conditions (Table 4 and Appendix Table 2). All feed quality traits ADF, NDF, ADL, OM, IVOMD, Me, and CP were significantly affected by accessions, growing seasons and moisture conditions and the interactive effect of accessions. Except for traits like ADL, IVOMD and Me, all nutritional traits were significantly affected by the interaction effect of accessions and growing seasons. Higher values in CP and Me were found during the dry season under severe water stress conditions while lower mean value was recorded under MWS during both wet and dry seasons (Figure 4A, 3B, 3C and 3D). Lower values in neutral detergent fibre and acid detergent lignin were recorded and higher mean value in ADL was recorded under MWS during the dry season and lower values in NDF and ADL were recorded under both moderate and severe water stress conditions during the dry season versus wet season.

Table 3. Mean Square from combined analysis of Agronomic and Nutritional Traits for Napier grass Accession evaluated Under Different Moisture Condition at Bishoftu During 2017-2020

Source of variation	Accession	**Season (Dry/wet)**	**MC (mws/sws)**	**Accessions *Season**	**Accessions*MC**	**Accession*Season* MC**	**Error**	**CV (%)**
				Phenotypic				
PH (cm)	1625.6***	1739836.24***	33.3ns	1155.9***	2.86ns	1.6ns	337.8 ns	**15.3**
LL (cm)	3352.8***	1279913.2***	128.5ns	339.1ns	16.1ns	78.2ns	17.8ns	20.9
LW (cm)	440.5***	672.4***	216.6***	63.9ns	1.79ns	219.26***	1.56ns	13.7
TN	73052***	5588928.1***	9176.69***	15658.5ns	504ns	197.55ns	458.5ns	26.8
TFW (t/ha)	4821.9***	1705197***	1.21ns	3191***	7.9ns	59.8ns	4.1ns	19.02
TDW (t/ha)	266.5***	81826.2***	47.4ns	169.1***	0.29ns	5.15ns	0.18ns	17.5
				Nutritional				
NDF (%)	40.33***	20606.7***	867.2***	21.1***	3.1ns	3.2ns	6.9 ns	14
ADF(%)	38***	30643.29***	2752.77***	15.8***	7.1ns	3.8ns	11.08 ns	8.61
ADL (%)	0.8ns	1448.5***	8.9***	0.26ns	0.06ns	0.06ns	0.6 ns	24.3
OM (%)	28.5***	638.4***	59.04***	12.99***	3.1ns	2.5ns	3.1 ns	12.1
CP (%)	45.8***	115.7***	3256.8***	19.1***	10.87**	3.9ns	8.2 ns	23.4
IVOMD (%)	26.4***	6033.75***	2232.3***	10.26ns	7.8ns	3.3ns	10.1 ns	15.67
Me (%)	0.36***	154.3***	24.6***	0.15ns	0.15ns	0.07ns	0.2 ns	16.1

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), TN= tiller number (mm), TFW= total fresh weight (gr), TDW = total dry weight (g), NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter, MC=moisture condition, mws=moderate water stress condition, sws=severe water stress condition

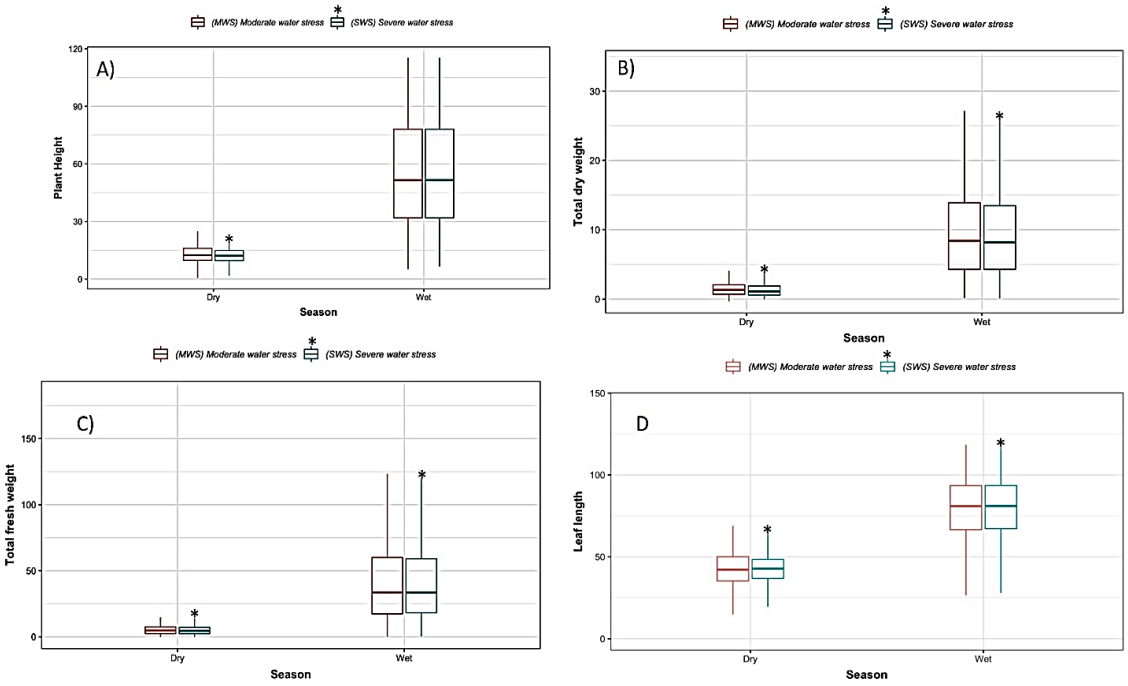


Figure 3. Response of agronomic traits to growing seasons and soil moisture conditions

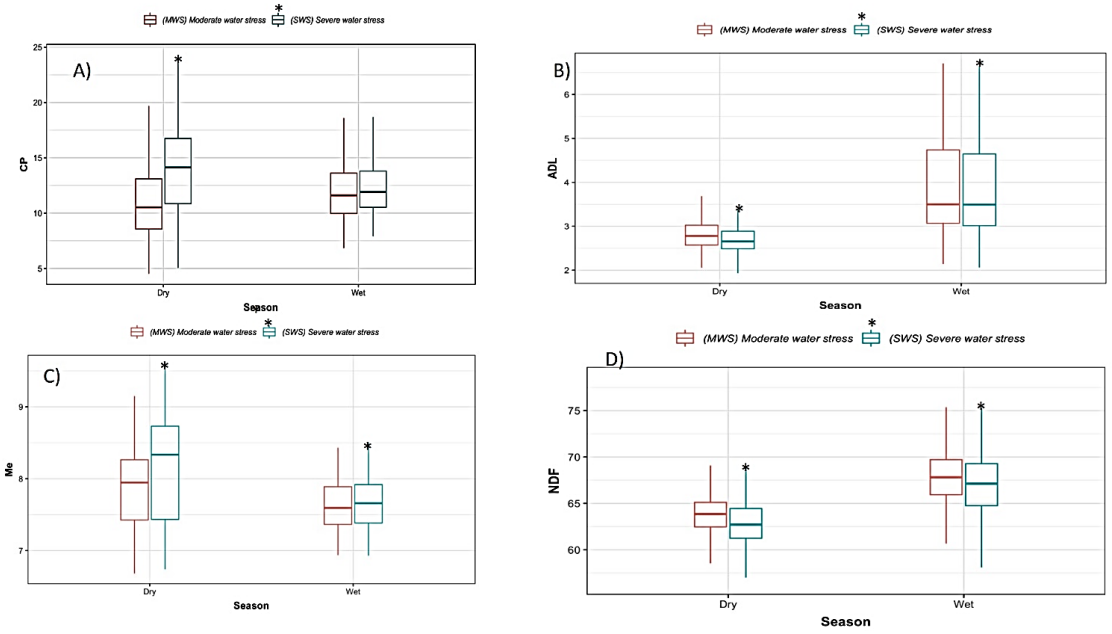


Figure 4. Response of nutritional traits to growing seasons and soil moisture conditions

4.1.2. Inter-Trait Correlation of Agronomic and Nutrition Traits

The correlation coefficient (R-values) was computed to determine the relationship between and among agronomic and nutritional traits as described in (Figure 5A and Appendix Table

3). For example, PH showed a strong positive correlation (*P<0.01*) with TFW, TDW, LL and LW. There was also a significant and strong positive correlation between TFW and TDW. On the other hand, TN depicted a weak negative association with PH, LL, and LW. Among nutritional traits, NDF showed a significant and positive correlation with ADF, ADL and OM but a significant and negative correlation with CP, IVOMD and Me. In addition, CP exhibited a significant and positive correlation with IVOMD and Me. There was no strong association between OM and other nutritional traits (Figure 5B and Appendix Table 3).

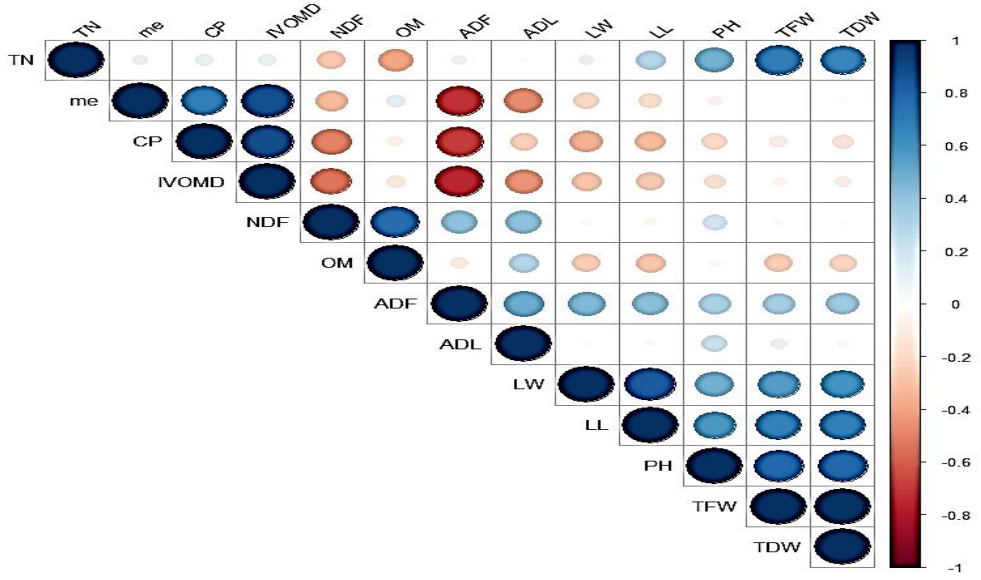


Figure 5. Correlation Analysis Between and Among Agronomic and Nutritional Traits

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), TN= tiller number (mm), TFW= total fresh weight (gr), TDW = total dry weight (g), NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter

4.1.3. Principal Component and Cluster Analysis

To determine the largest contributing traits, principal component analysis was done (Figure 6A). As the PCA the scree plot (Figure 6B) four principal components (PC1 to PC4) had eigenvalues greater than one and eigenvalues make a straight line after the fourth component. These retained first four components accounted for 86.3 % of the total variation among accessions for the studied agronomic and nutritional traits (Table 5). In the first two principal components (PCs) total of 64.1 %, explained variances PC1 (38.1%) and PC2 (26%) was determined. All the agronomic traits showed a similar maximum correlation with the (PC1) which were ordinated in the same dimension and found a strong positive correlation among traits. However, nutritional traits were distributed in different components i.e., IVOMD, CP, and Me were ordinated in the second component which contains the second greatest variation and negatively correlated with the rest of nutritional traits but OM, ADL, ADF, and NDF were appeared in the fourth component and positively correlated with each other but negative correlation with IVOMD, CP and Me (Figure 6A, Table 5).

Hierarchal clustering was done for grouping accessions using agronomic and nutritional traits (Figure 6 C). A total of 31, 19, and 24 accessions were grouped in the first, second and third clusters, respectively. First clusters were sub-grouped into two clusters and most of the CNGPL-EMBRAPA elite lines and ILRI accessions are captured in the first cluster. BAGCE accessions were only grouped over the first two clusters and the third cluster contains only ILRI collection with one distantly related accession of the CNPGL-EMBRAPA elite line. Clustering all the accessions based on phenotypic and nutritional traits scattered into different groups regardless of background or collected origin.

Table 4. Eigenvectors and eigenvalues of the first 4 principal components for 13 different agronomic and nutritional traits of 86 Napier grass accessions

Traits	PC1	PC2	PC3	PC4
TFW	0.75	0.58	0.23	-0.10
PH	0.72	0.35	0.39	-0.04
TDW	0.77	0.54	0.23	-0.04
TN	0.38	0.61	0.08	-0.53
LW	0.71	0.22	-0.16	0.53
LL	0.76	0.35	-0.12	0.39
NDF	0.31	-0.67	0.59	0.16
ADF	0.79	-0.40	-0.18	-0.13
ADL	0.35	-0.48	0.28	-0.49
OM	-0.15	-0.52	0.78	0.22
IVOMD	-0.25	-0.42	0.58	0.32
CP	-0.64	0.57	0.20	-0.14
Me	-0.56	0.61	0.40	0.23
Eigenvalue	4.9	3.5	1.6	1.1
Precent cumulative variance	37.7	65.0	77.3	86.1

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), TN= tiller number (mm), TFW= total fresh weight (gr), TDW = total dry weight (g), NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter,

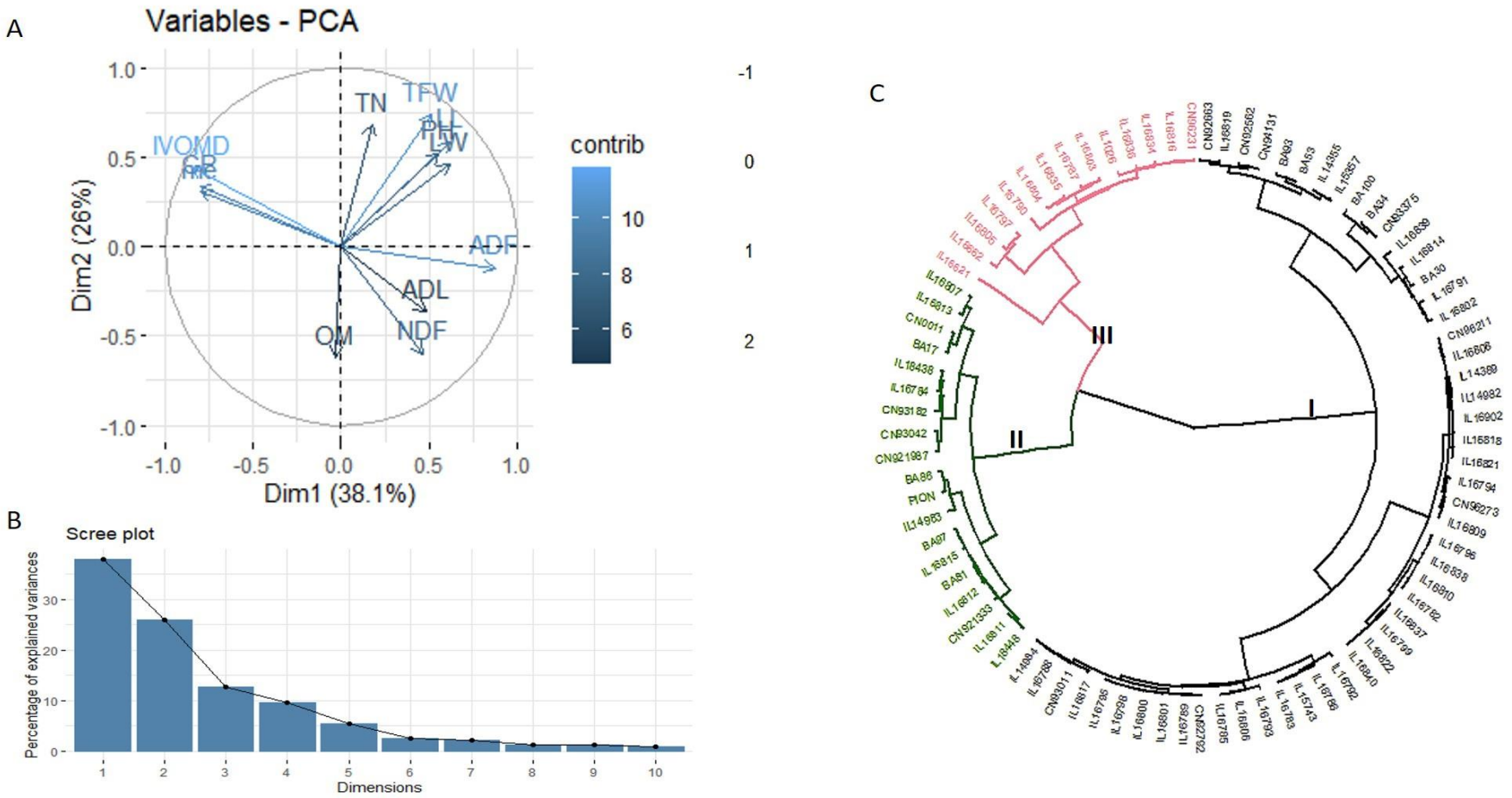


Figure 6. Scatter plot (a), Scree plot of the PCA (b) and Cluster dendrogram of Napier grass accessions based on agronomic and nutritional traits (c).

4.2. SNP Discovery and GWAS

4.2.1. Genome-Wide SNP Discovery, Its Distribution and Assembled Chromosome

Whole-genome sequencing of 109 Napier grass accessions generated a total of 108,957,694 variants (SNPs and Indels). Of the total variants about 90,803,632 were SNPs and 19,654,799 were indels (Table 6). After hard filtering, about 1,129,470 SNPs were kept for subsequent genotyping but Indels were not included for downstream analysis because of enough SNPs. Based on the filtered SNPs a variant was detected at every 1683 bases. An accession 16621, showed below-average mapping quality hence removed from subsequent downstream analysis.

Table 5. SNP Filtering Steps

SNP filtering steps	SNPs retained	SNPs discarded at each stage of filtering	SNPs discarded (%)
Total No of variants (SNPs & INDELS)	90,803,632		
Bi-allelic and polymorphic SNPs	68,745,980	22,057,652	20.2%
FMT/DP>10	3,296,426	65,449,554	95.2%
FMT/ 10> DP <300	3,186,455	109,971	3.3%
FMT/GQ>20	3,183,164	3,291	0.1%
MAF>0.02 & F_MISSING<=0.85	2,638,827	544,337	20.6%
prune -l 0.6 -w 1000	1,975,261	662,881	25.1%
prune -l 0.2 -w 1000	1,129,470	1,508,672	76.4%

After filtering, the largest SNPs were found in chromosome B01 followed by chromosome B02 and the smallest SNPs were mapped on chromosome A06 followed by chromosome A07. From total identified SNPs higher (716,080 SNPs) and lower (413,390) were mapped on B and A chromosomes, respectively (Figure 7).

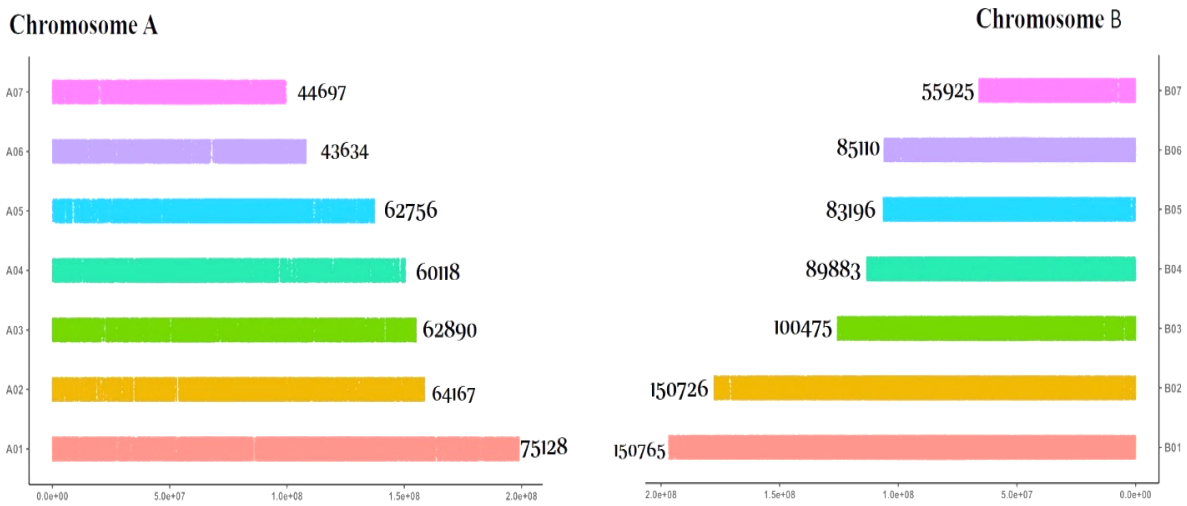


Figure 7. Genome-wide single nucleotide polymorphisms (SNP) density distribution over fourteen chromosomes (A and B) of Napier Grass

Most of the filtered SNPs were located at intergenic regions (85%) and 10,374 (0.8%) were in the Exon region of the genome. Among the identified SNPs, the rate of transition was much higher than transversion and the ratio of Ts/Tv was 3.09. A total of 180 unique gene IDs were also detected among that filtered SNP. SNPs (99.13%) were found modifier based on its impact effect while 49.96 % were found silent based on effect of SNPs by its functional class (Table 7). SNP density (0.085 %) was found from the total size of 177,737,733kb on chromosome B02 which has a lower size than the B01. The lowest SNP density (0.0037%) was recorded for chromosome A01 but it has a higher genome size than the rest of A sub-genomes. In general, SNP density across assembled chromosomes was not associated with its respective size (i.e from a higher size smaller SNPs were identified and vice-versa (Table 8).

Table 6. Number of SNP effects by Type, Region, Impact, and Functional Class

Type	Count	Percent	**Region**	Count	Percent
Downstream_gene_variant	64,122	5.12	Downstream	64,122	5.12
Initiator_codon_variant	1	0	Exon	10,426	0.83
Intergenic_region	1,065,120	85.02	Intergenic	1,065,120	85.02
Intron_variant	53,405	4.26	Intron	53,405	4.26
Missense_variant	5,003	0.40	Splice_site_acceptor	18	0.00
Missense_variant+splice_region_variant	70	0.01	Splice_site_donor	14	0.00
Splice_acceptor_variant+intron_variant	18	0.00	Splice_site_region	462	0.04
Splice_donor_variant+intron_variant	14	0.00	Transcript	6	0
Splice_region_variant+intron_variant	398	0.03	Upstream	59,155	4.72
Splice_region_variant+stop_retained_variant	7	0.00	**Number of effects by impact**		
Splice_region_variant+synonymous_variant	57	0.01	High	210	0.02
Start_lost	5	0	Low	5,643	0.45
Stop_gained	166	0.01	Moderate	5,073	0.41
Stop_gained+splice_region_variant	4	0	Modifier	1,241,802	99.13
Stop_lost+splice_region_variant	3	0	**Number of Effects by functional class**		
Synonymous_variant	5,180	0.41	Missense	5,082	48.42
Upstream_gene_variant	59,155	4.72	Nonsense	170	1.62
Base changes (SNPs)	**Ts/Tv (transitions / transversions)**		Silent	5,244	49.96
A C G T	Transitions	49,997,325	Missense / Silent ratio:	0.9691	
A 0 24,581 104,960 44,535	Transversions	16,191,553			
C 44,211 0 25,906 320,322	Ts/Tv ratio	3.0879			
G 319,566 26,140 0 44,291					
T 44,578 106,055 24,325 0					

Table 7. Total length, SNPs density percent and SNP variant rate over mapped A and B chromosome of Napier Grass genome

Chromosomes	Length (kb)	Total SNPs (#)	SNP Density (kb)	SNP density (%)	SNP Variants rate
A01	199,064,672	75,128	2.65	0.0377	2,649
A02	158,795,698	64,167	2.47	0.0404	2,475
A03	155,160,916	62,890	24.66	0.0405	2,466
A04	150,585,890	60,118	2.50	0.0399	2,506
A05	137,443,833	62,756	2.19	0.0457	2,190
A06	108,239,444	43,634	2.48	0.0403	2,482
A07	99,749,506	44,697	2.23	0.0448	2,230
B01	196,755,181	150,765	1.31	0.0766	1,305
B02	177,737,733	150,726	1.18	0.0848	1,179
B03	125,700,457	100,475	1.25	0.0799	1,251
B04	113,328,846	89,883	1.26	0.0793	1,261
B05	106,417,498	83,196	1.28	0.0782	1,279
B06	106,011,349	85,110	1.25	0.0803	1,245
B07	66,044,712	55,925	1.18	0.0847	1,181
Total	1,901,035,735	1,129,470	1.683	0.0594	1,683

4.2.2. Population Structure and Cluster Analysis

About 1,129,470 SNPs were used for clustering and population structure analysis. The PCA analysis revealed a mixed trend where ILRI accessions were scattered into all the coordinates (Figure 8A). Interestingly, most CNPGL and BAGCE accessions were captured in the first and second coordinates. There were some outliers from ILRI accessions in the fourth coordinate and similar contributions were observed for all the ordinates. Both, Super Napier, and PIONEIRO varieties contributed to the second ordinate. The hierarchical cluster analysis showed there were two main clusters (A and B) into which accessions were grouped based on their dissimilarity matrix (Figure 8B). The distribution of the ΔK (Figure 8D) shows a clear optimum cluster peak at K=2 indicating that the presence of two major groups with each have further subclusters.

A total of 49 accessions out of 108 were grouped in the first cluster and the rest were grouped in the second cluster. ILRI and CNPGL accessions were equally captured in both Clusters but most of CNPGL were captured in cluster A and are aggregated non distantly into a similar sub-cluster. The three USA accessions did not cluster together. Cluster and structure analysis (Figure 8B and C) showed no clear pattern based on their country of origin and an admixture of accessions were observed which grouped into the different clusters and sub-clusters regardless of their country of origin.

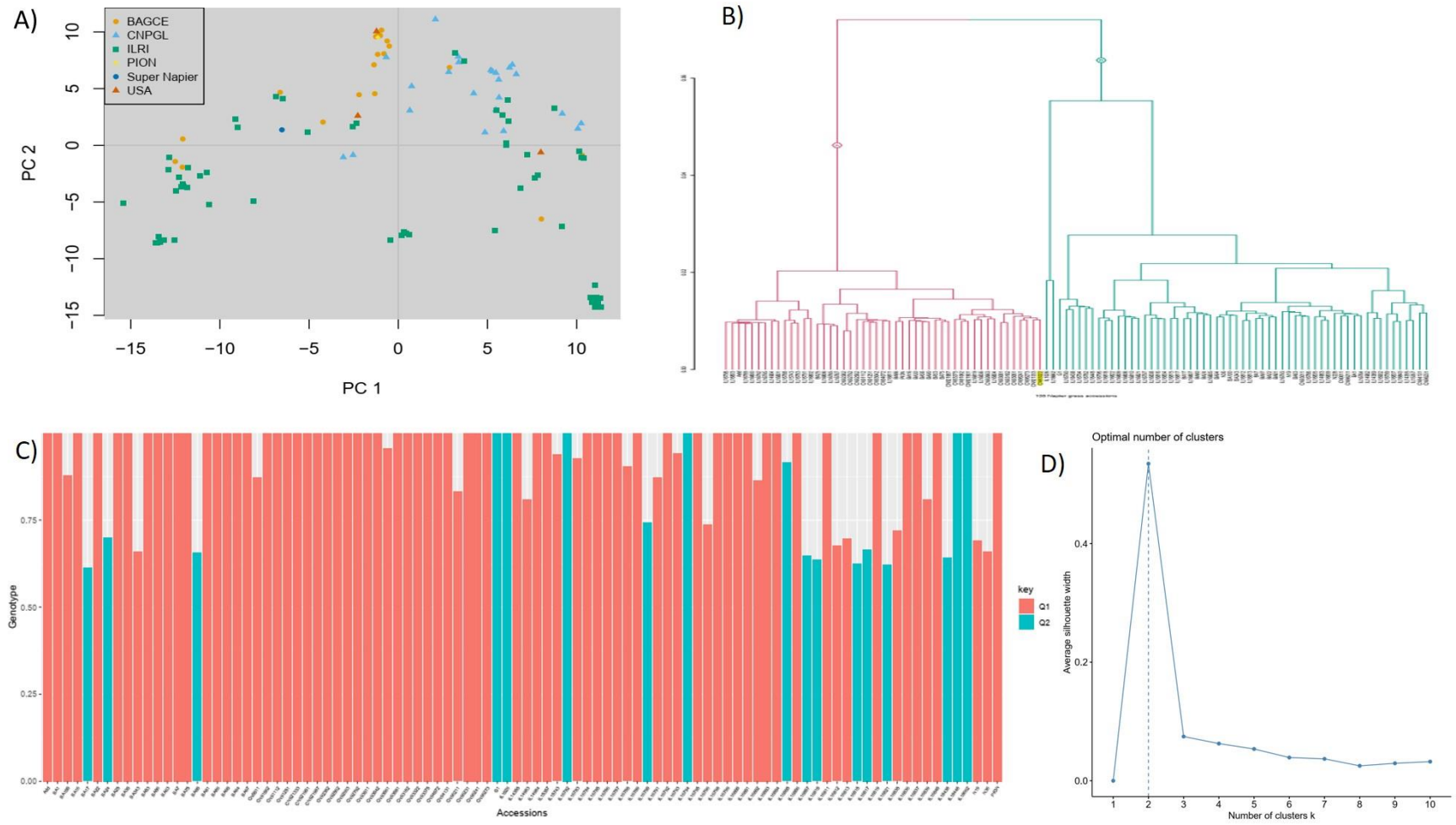


Figure 8. Principal coordinate analysis (A), Cluster (B) and structure (c) and Optimal ΔK (d) of Napier grass accessions

4.3. Marker-Traits Associations

4.3.1. Agronomic and Nutritional traits

Eighty-four accessions were phenotyped for two years, under two soil water conditions, and these data were combined with genotyping data for GWAS analysis. A total of 1,129,470 SNPs were used for GWAS analysis. More than 100 SNPs were significantly correlated - log10 (p-value) ≥ 5.0) threshold using a Circulating Probability Unification model (FarmCPU), for both agronomic and nutritional traits (Table 9 and Appendix Table 4). For example, 21 SNPs were significantly associated (Figure 9 A and B) with PH during the dry season (six SNPs under SWS condition which are mapped at chromosome A02 (1SNP), A06 (1SNP), B01 (1SNP), B03 (2SNP), B06 (1SNP) and two SNPs under MWS condition which mapped at chromosome A05 (1 SNP), and A06 (1SNP). Similarly, SNPs that are putatively associated with PH during the wet season under MWS were located at chromosome A02 (1SNP), A06 (1 SNP), A07 (1 SNP), B03 (2 SNPs), B04 (1 SNPs) and B05 (1 SNP). SNPs mapped at chromosome B03 were strongly correlated with PH under SWS during the dry season. During wet season SNP that mapped on chromosome B04 under MWS condition and SNP at chromosome A02 (1SNP) under SWS have a strong correlation with PH.

There were also 14 SNPs putatively associated (Figure 10 A and B) with TFW during the wet season (five SNPs under MWS condition which are mapped at chromosome A04 (2 SNPs), A05 (1SNP), B04 (1SNP), B07 (1SNP) and four SNPs under SWS condition which are mapped at chromosome A04 (2 SNPs), and A07 (2 SNPs). Besides, during the dry season, in MWS condition, SNPs associated with TFW were located A04 (1SNP), A05 (1 SNP), B04 (1 SNP) and B07 (1SNP) but there were no SNPs that were significantly associated with TFW under SWS condition during the dry season. There were also significantly correlated SNPs with the rest of the agronomic traits measured in this study

(Table 9). Similarly, GWAS analysis for nutritional traits identified SNPs that were significantly associated with measured traits (Table 9). SNPs putatively associated with CP were located at chromosome A04 (1 SNP) and A02 (1 SNP) under MWS and SWS conditions, respectively during the dry season.

Moreover, in both seasons there were a total of 13 SNPs (seven during the wet season and six during the dry season) that were significantly associated with ADL (Figure 11 A and B). During the wet season, six total identified SNPs that pass - log10 (p-value) $\geq$ 5.0) were found under the MWS condition while a SNP was found under the SWS condition. These significantly associated SNPs for ADL were located on chromosome A03 (3 SNPs), A04 (1SNP), B02 (1SNP), B06 (2SNPs) under MWS condition while A01 (1SNP) under SWS condition of WS. SNPs located at one from chromosome A03 and A04 were found correlated with ADL under the MWS condition of the wet season.

Similarly, during the dry season, a total of six SNPs (two SNPs under MWS condition and four under SWS condition) were significantly linked with ADL. These SNPs were located B03 (1SNP), B05 (1SNP) under MWS condition and four were located at chromosome A01 (1SNP), A06 (1SNP) B01 (1SNP), B02 (1SNP) and B04 (1SNp) under SWS condition. Several SNPs were also detected for the other feed quality traits in the study (Table 9). Interestingly, most of the SNPs identified in the present study were shared by different traits or treatment conditions. For example, SGWHAORA00000005_58573147 was a significantly associated variant in both LL and TFW traits. Similarly for nutritional traits, SNP SGWHAORA00000013_36372759 was shared among ADF, IVOMD and Me traits.

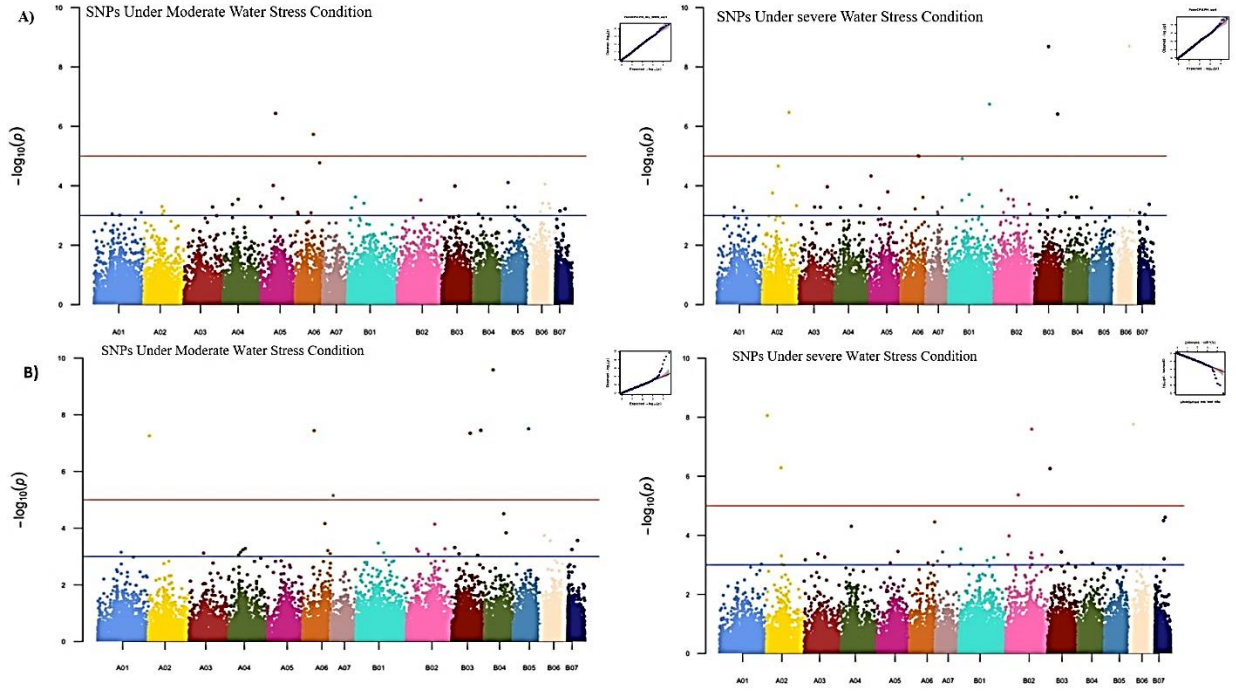


Figure 9. Manhattan plots showing SNPs Significantly Associated (FarmCPU Method) with Plant Height During Dry (A) and Wet (B) seasons

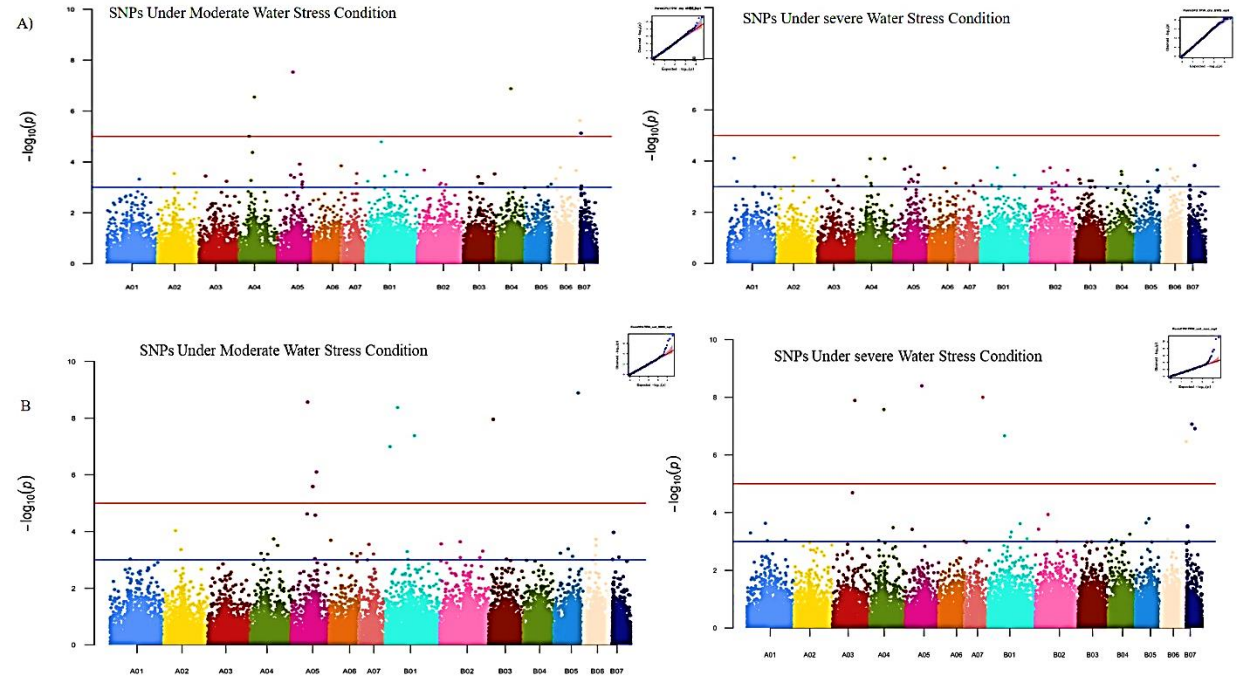


Figure 10. Manhattan plots showing SNPs Significantly Associated (FarmCPU method) with Total Fresh Weight During dry (A) and Wet (B) Seasons

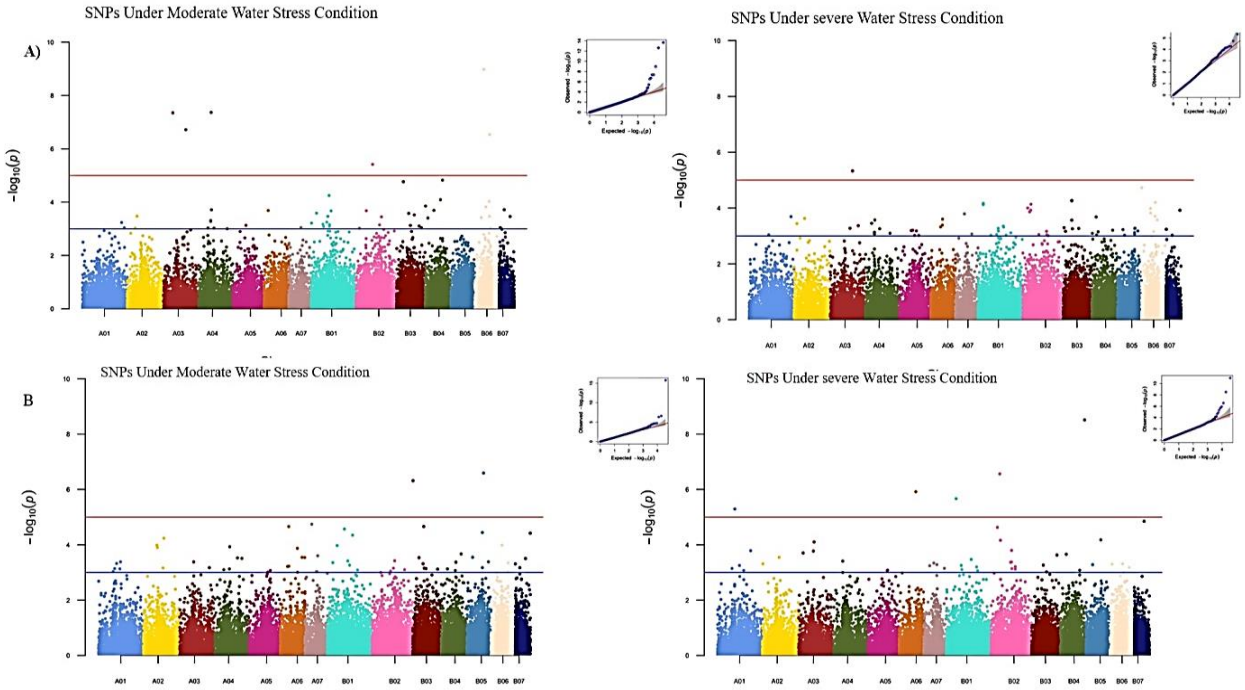


Figure 11. Manhattan plots showing SNPs Significantly Associated (FarmCPU method) with Acid Detergent Lignin During dry (A) and Wet (B) Seasons

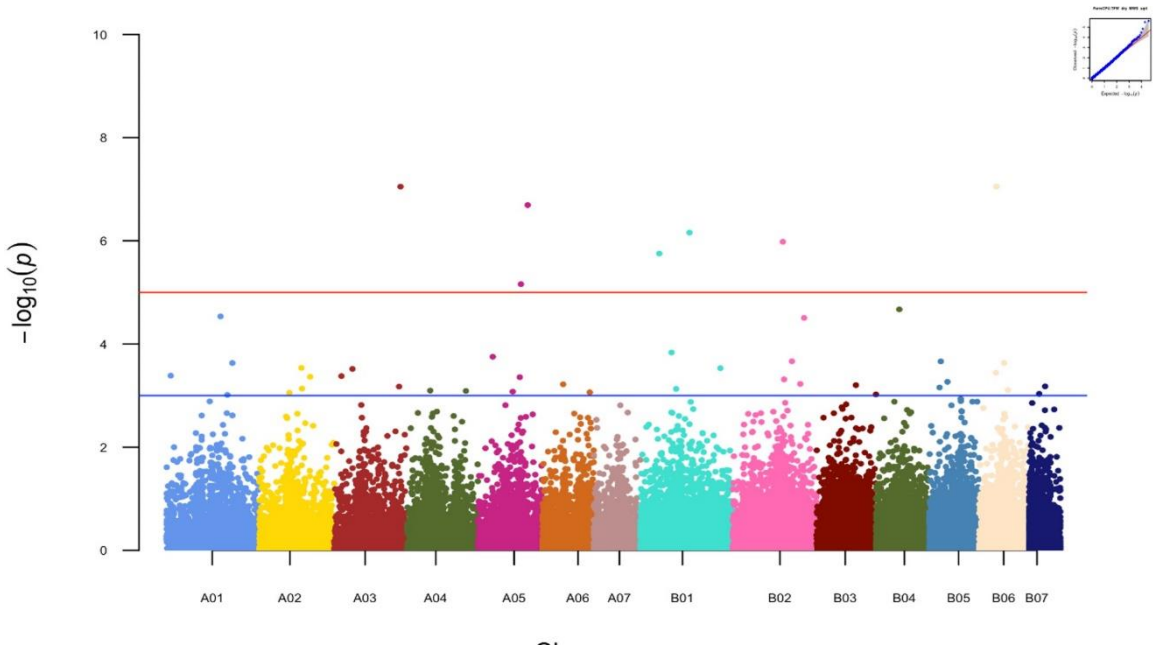


Figure 12. Manhattan plots showing SNPs Significantly Associated (FarmCPU method) with Acid Detergent Fibre During Dry Seasons under severe water stress condition.

Table *8*. Significantly associated markers with Plant Height, its mapped chromosome location, allele, and position that passed threshold level of Farm CPU (P < 1.00E-05).

SNP	Traits	Season	Treatment	Allele	Chromosome	Position	P.value	MAF	FDR_Adjusted_P.values	effect
SGWHAORA00000005	LL	Dry	MWS	A/G	A05	58573147	0.0	0.139	0.00000	-0.3749
SGWHAORA00000005	LL	Wet	MWS	A/G	A05	58573147	0.00	0.139	0.00000	-0.3749
SGWHAORA00000005	LL	Wet	SWS	A/G	A05	58573147	0.00	0.139	0.01202	-0.2261
SGWHAORA00000005	TFW	Wet	MWS	A/G	A05	58573147	0.00	0.139	0.00112	-0.5439
SGWHAORA00000005	TFW	Wet	SWS	A/G	A05	58573147	0.00	0.139	0.00000	-0.6746
SGWHAORA00000009	LL	Dry	MWS	G/A	B02	40040366	0.00	0.380	0.00004	0.16459
SGWHAORA00000009	LL	Wet	MWS	G/A	B02	40040366	0.00	0.380	0.00004	0.16459
SGWHAORA00000014	LL	Dry	MWS	G/A	B07	3247644	0.00	0.481	0.00040	0.52199
SGWHAORA00000014	LL	Wet	MWS	G/A	B07	3247644	0.00	0.481	0.00040	0.52199
SGWHAORA00000013	LL	Dry	MWS	C/G	B06	47707055	0.00	0.443	0.00065	0.41998
SGWHAORA00000013	LL	Wet	MWS	C/G	B06	47707055	0.00	0.443	0.00065	0.41998
SGWHAORA00000012	LL	Dry	MWS	A/C	B05	17150204	0.00	0.070	0.00070	0.25522

Table 8. Continued

SGWHAORA00000012	LL	Wet	MWS	A/C	B05	17150204	0.00	0.070	0.00070	0.25522
SGWHAORA00000013	LL	Dry	MWS	C/A	B06	105448364	0.00	0.038	0.00093	-0.3333
SGWHAORA00000013	LL	Wet	MWS	C/A	B06	105448364	0.00	0.038	0.00093	-0.333
SGWHAORA00000013	TFW	Wet	MWS	C/A	B06	105448364	0.00	0.038	0.02255	-0.833
SGWHAORA00000013	TFW	Wet	SWS	C/A	B06	105448364	0.00	0.038	0.01075	-0.708
SGWHAORA00000011	LL	Dry	MWS	T/C	B04	67364239	0.00	0.076	0.00889	0.20491
SGWHAORA00000011	LL	Wet	MWS	T/C	B04	67364239	0.00	0.076	0.00889	0.20491
SGWHAORA00000006	LL	Dry	MWS	T/C	A06	56903089	0.00	0.133	0.02021	-0.1115
SGWHAORA00000006	LL	Wet	MWS	T/C	A06	56903089	0.00	0.133	0.02021	-0.1115
SGWHAORA00000011	LL	Dry	MWS	C/T	B04	65799675	0.00	0.038	0.02173	0.26146
SGWHAORA00000011	LL	Wet	MWS	C/T	B04	65799675	0.00	0.038	0.02173	0.26146
SGWHAORA00000010	PH	Wet	MWS	A/G	B03	48939934	0.00	0.171	0.00001	0.35453
SGWHAORA00000010	PH	Wet	SWS	A/G	B03	48939934	0.00	0.171	0.00115	0.25306
SGWHAORA00000001	PH	Wet	MWS	A/T	A01	92750473	0.00	0.076	0.00003	-0.3858

Table 8. Continued

SGWHAORA00000001	PH	Wet	SWS	A/T	A01	92750473	0.00	0.076	0.03421	-0.246
SGWHAORA00000013	PH	Wet	MWS	T/C	B06	45734009	0.00	0.076	0.00015	-0.466
SGWHAORA00000013	PH	Wet	SWS	T/C	B06	45734009	0.00	0.076	0.00000	-0.668
SGWHAORA00000008	PH	Wet	MWS	C/T	B01	156728589	0.00	0.259	0.00101	-0.3058
SGWHAORA00000008	PH	Wet	SWS	C/T	B01	156728589	0.00	0.259	0.00033	-0.33
SGWHAORA00000014	PH	Wet	MWS	T/C	B07	37004642	0.00	0.241	0.02749	-0.1579
SGWHAORA00000014	PH	Wet	SWS	T/C	B07	37004642	0.00	0.241	0.03421	-0.168
SGWHAORA00000004	TDW	Dry	MWS	C/T	A04	112701449	0.00	0.108	0.14569	-0.238
SGWHAORA00000004	TFW	Dry	MWS	C/T	A04	112701449	0.00	0.108	0.06108	-0.480
SGWHAORA00000001	TDW	Dry	MWS	T/C	A01	26467349	0.00	0.070	0.14569	-0.284
SGWHAORA00000001	TFW	Dry	MWS	T/C	A01	26467349	0.00	0.070	0.06108	-0.5739
SGWHAORA00000004	TDW	Wet	MWS	T/C	A04	59427440	0.00	0.032	0.21574	-1.189
SGWHAORA00000004	TDW	Wet	SWS	T/C	A04	59427440	0.00	0.032	0.17634	-1.199
SGWHAORA00000004	TFW	Wet	MWS	T/C	A04	59427440	0.00	0.032	0.00358	-1.211

Table 8. Continued

SGWHAORA00000004	TFW	Wet	SWS	T/C	A04	59427440	0.00	0.032	0.00027	-1.158
SGWHAORA00000011	TFW	Wet	MWS	T/C	B04	57738396	0.00	0.057	0.00251	-0.832
SGWHAORA00000011	TFW	Wet	SWS	T/C	B04	57738396	0.00	0.057	0.00000	-1.095
SGWHAORA00000013	ADF	Dry	SWS	A/G	B06	36372759	0.00	0.397	0.00169	-0.073
SGWHAORA00000013	IVOMD	Dry	SWS	A/G	B06	36372759	0.00	0.397	0.00000	0.07153
SGWHAORA00000013	ME	Dry	SWS	A/G	B06	36372759	0.00	0.397	0.00000	0.02217
SGWHAORA00000003	ADL	Wet	MWS	C/G	A03	98130331	0.00	0.276	0.00121	0.01206
SGWHAORA00000003	ADL	Wet	SWS	C/G	A03	98130331	0.00	0.276	0.17695	0.02733
SGWHAORA00000002	CP	Dry	SWS	G/C	A02	153925499	0.00	0.051	0.15871	0.28497
SGWHAORA00000002	ME	Dry	SWS	G/C	A02	153925499	0.00	0.051	0.00000	0.03524
SGWHAORA00000009	IVOMD	Dry	SWS	T/G	B02	93527062	0.00	0.154	0.00759	-0.021
SGWHAORA00000009	ME	Dry	SWS	T/G	B02	93527062	0.00	0.154	0.01188	-0.00
SGWHAORA00000004	IVOMD	Dry	SWS	T/G	A04	62759479	0.00	0.154	0.02607	0.01941
SGWHAORA00000004	ME	Dry	SWS	T/G	A04	62759479	0.00	0.154	0.05161	0.00714
SGWHAORA00000006	NDF	Wet	MWS	C/A	A06	52372976	0.00	0.032	0.22291	0.14356
SGWHAORA00000006	NDF	Wet	SWS	C/A	A06	52372976	0.00	0.032	0.21893	0.14212

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), ST= stem thickness (mm), TN= tiller number (mm), IL= internode length, TFW= total fresh weight (gr), TDW = total dry weight (g), LSR=leaf-stem-ratio, NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter

5. DISCUSSIONS

5.1. Variability in Agronomic and Nutritional Traits

In the current study, the result revealed growth and forage biomass yield of Napier grass was significantly affected by accessions, moisture conditions, and seasons (Table 4). This result was in agreement with findings reported by Habte *et al.* (2020), who found that the forage yield of Napier grass was significantly different across genotypes and growing seasons. Consistently similar results also were reported from the study conducted by Shanableh *et al.* (2016), who found that growth and forage biomass yield of pearl millet were significantly different across accessions. Growth and yield traits like, PH, TFW, and TDW were higher during the wet season while lower during the dry season (Figure 3). Dinkale *et al.* (2021) reported that Dry matter and fresh biomass yield were high during the rainy season as was in the present study. This study revealed a significant difference in measured agronomic traits, across accessions, which was also reported by Zewdu (2005), in which a similar result in a study that included most of the accessions in the present study but the experiment was carried out in a different location. The above results highlight the fact that maximum yield can be harnessed from Napier grass if there is a continuous supply of water during production. ILRI accessions 16801 and 16804 were recently released varieties for biomass yield (Tulu *et al.*, 2021) but some of the accessions in the present study performed as good or better, in terms of PH, TFW and TDW highlighting the possibility of further improvement of released varieties.

Nutritional traits of Napier grass were also significantly different, among the accessions, implying inherent polymorphism, in terms of feed quality traits, due to their genetic background (Table 4 and Figure 4). In the present study, higher mean CP content was observed in dry seasons and under SWS in wet season conditions. This result was in

agreement with the study conducted by Kebede *et al.* (2017), who found that CP yield, digestibility were higher at lowland (with high temperature) than highlands (more wet conditions) for different Napier grass accessions studied. A similar study on *Brachiaria* spp. cultivars showed higher CP content under dry conditions as was shown in the present study (Garay *et al.*, 2017). Comparatively, nutritional traits were, more importantly, responding to the interactive effects than agronomic traits (Table 4) implying that nutritional content can be more determined by the interactive effect of accessions with stress conditions and this finding agreed with the study conducted (Kebede *et al.*, 2016).

In the present study, CP was higher under SWS conditions during the dry season and lower during the wet season which highlights soil moisture plays a key role in the nutritional qualities of Napier grass. This result suggests that limited soil moisture has a positive impact on nutritional values like CP in Napier grass and a similar finding was reported by Bahreininejad (2019), who recorded higher mean CP under drought stress conditions. Likewise, higher values in Me and IVOMD, during low moisture conditions, were observed which is in agreement with the studies conducted by Habte *et al.* (2020); and Bahreininejad (2019). All nutritional traits were significantly affected by the cumulative effect of accessions and growing seasons and these results were consistent with the study conducted by (Habte *et al.*, 2020; Maleko *et al.*, 2019; Mwendia *et al.*, 2017). Hence, livestock farmers should be made aware of this seasonal fluctuation in feed quality traits in Napier grass and should supplement their livestock accordingly.

5.2. Inter Agronomical and Nutritional Trait Association.

Correlation analysis indicated PH was significantly and positively correlated with LL, LW TFW and TDW, indicating that these traits can be improved simultaneous (Figure 5). This finding was consistent with the report made by (Rahul, 2017). Correspondingly, there was a

significant association among nutritional traits. For example, NDF showed a significant and positive correlation with ADF, ADL and this finding was consistent with a report from Habte *et al*. (2020). Similarly, ADF and NDF exhibited a significant and strong negative correlation with CP, IVOMD and Me which might be indicating that fibre content significantly affects factors of nutritional traits and palatability of Napier grass; similar studies were also reported by (Maleko *et al*., 2019). Also, in this study nutritional traits that are positively and strongly correlated will be a promising potential to improve those traits i.e., breeding to improve one trait will improve other traits that positively correlated with the trait of interest. Similar reports were found that improving one trait can improve other traits which have a positive correlation with traits of interest (Henkin *et al.*, 2011).

5.3. Variability of Napier Grass collections under Different Moisture Condition

5.3.1. Variability of Napier grass Collections Based on Phenotypic and Nutritional Traits

This study revealed that there was phenotypic, nutritional, and genetic variability among global Napier grass collections (Figure 6 and 8). Principal component analysis categorized traits into three coordinates and the first four PCAs describes 86.4 % of cumulative explained variation and the biplot was drawn using two major ordinates explaining cumulative of 64% variation indicating that their relations among the traits (Figure 6 a and c). Traits in the first components were greater contribution for the variation and strong positive association between traits indicating that improving for these traits will be promising further Napier grass breeding. A similar finding was reported by (Rahul, 2017).

Hierarchal clustering based on measured traits grouped accessions into three clusters regardless of their geographical origins. This finding was in agreement with the study conducted by Pattanashetti *et al.* (2015), who studied Napier grass collections at ICRISAT

India which were clustered irrespective of the source of country origin. In the cluster analysis, 51 accessions (38 ILRI, 8 CNPGL and 5 BAGCE accessions) were grouped into the first cluster. Interestingly, only ILRI accessions were grouped into the second Cluster, 14 of them and 9 ILRI accessions, 5 CNPGL, 4 BAGCE and PIONEIRO accessions were categorized into the third cluster. These groupings regardless of country of the collection might be due to germplasm exchange between countries and a similar finding was reported by (Wanjala *et al.*, 2013).

5.3.2. Genome-wide SNP Markers for Napier grass

When curated reads were mapped against the recently published Napier grass genome (Yan *et al.*, 2021), more than 100 million variants were detected of which 90,803,632 were SNPs and 19,654,799 were Indels. Previous GBS studies on Napier grass by Muktar *et al.* (2019) and Paudel *et al.* (2018) generated only 100k SNP and Indel markers but the present study exceeds this threshold significantly, with one million-plus hard filtered SNPs because of whole genome sequencing approach cover wider genomic regions. The distribution of filtered SNPs across chromosomes varies considerably and the highest number of SNPs was recorded for the B sub-genome chromosomes, 150,765, 150,726, 100,475, B01 and 02, 03, respectively (Figure 6). A GBS study on Napier grass also showed a similar result where the highest SNPs were mapped in the B sub-genome (Muktar *et al.*, 2019). Among A sub-genome chromosomes, the higher SNP number was recorded for A01 as was reported by (Muktar *et al.*, 2019). Napier grass's A' chromosomes are homologous to A genomes of pearl millet (Gupta and Mhere, 1997) and the tools developed in this study can also play a role in key forage and feed species, pearl millet and hybrids originating from these two closely related species. This is the first study that generated genome-wide makers, a SNP at every 1,683 bases even after hard filtering, for Napier grass and these genomic tools will be

critical for advancing Napier grass breeding technology for its improvement and full domestication.

5.3.3. Clustering and Population Structure Based on Genome-Wide SNP markers

More than a million SNPs were shared among 108 Napier grass accessions; implying that there is polymorphism among these collections. This finding is consistent with the report made by Muktar *et al.* (2019), who found the presence of a significant quantity of variation between the ILRI collections with some distinctive features among EMBRAPA collections. Principal component analysis revealed that accessions were scattered into ordinates with no clear structure (Figure 8). This is expected for ILRI accessions as they were of global origin. Interestingly, most EMBRAPA elite lines (CNPGL) clustered into one quarter indicating similarity in their origin. In addition, USA accessions, three of them, clustered close to EMBRAPA accessions which were in agreement with finding from (Muktar *et al*, 2021). The whole-genome sequencing approach also singled out an accession (16621) with poor quality mapping to the reference genome which implies this accession is not the same species. A previous GBS study by Muktar *et al.*, (2019) reported a similar trend for this accession.

Structure analysis indicated there were delta K=2 optimal clusters which were consistent with cluster analysis result through Unweighted Pair Group Method with Arithmetic Mean (UPGMA) where accessions were grouped into two clusters (A and B) regardless of their country of origin (Figure 8 b and c). Each main hierarchical cluster contains 49 accessions and with each cluster, accessions were sub-grouped into a small cluster. A possible reason for the grouping of accessions irrespective of their geographic origin might be that populations were admixtured due to germplasm exchange across global regions. Similar

reports were made by (Muktar *et al.*, 2019; Negawo *et al.*, 2018; Tadelech, 2021; Wanjala *et al.*, 2013).

5.4. Genome-Wide Association Study

Association mapping of **SNPs with Agronomic and Nutritional Traits**

Genome-wide association studies (GWAS) have opened the door for systematic discovery of genetic factors for complex traits such as yield, disease and pest resistance, nutritional quality etc (Kaur *et al.*, 2021). While GWAS have provided new insights into genetic factors affecting traits of interest, these genetic variants only explain a small proportion of the phenotypic variance attributable to genetic factors (Manolio *et al.*, 2009). The large unidentified heritability can be partially explained by various factors including allelic heterogeneity, independent association of common SNPs or cumulative effects of rare variants in single loci (Elorbany *et al.*, 2022; García-Cañas *et al.*, 2014; Ward *et al.*, 2022).

In the present study, GWAS have successfully mapped thousands of loci associated with both agronomic and nutritional traits of Napier grass, in two seasons (dry and wet) and under two soil moisture conditions (MWS and SWS). One of the key complex traits affected by water stress was PH and it is regulated by multiple loci with small effects. The GWAS analysis has identified SNPs significantly associated with PH ($P < 1.00E-05$) under both dry and wet conditions of the trial. These SNPs were further checked for their significance under MWS and SWS (Figure 9-12). Previous field characterization of Napier grass accessions in Ethiopia, under irrigation conditions, recorded improved performance for PH than rainfed conditions (Faji *et al.*, 2022).

Therefore, the PH associated SNPs identified in the present study can be of great value in future selection programs to select high yielding Napier grass accessions. Biomass yield is one of the key traits in forage crops and a recent study comparing 9 perennial tropical forage

types of grass showed that Napier grass gives the highest dry matter yield per hectare (Faji *et al.*, 2022). In the present study SNPs significantly associated with TFW were recorded in MWS and SWS conditions, for the dry season. A study in pearl millet, which can hybridize with Napier grass and is thought to be one of the progenitors of Napier grass identified loci that significantly associate biomass yield and fresh weight (Habyarimana *et al*., 2020) and these loci were located at chromosomes 7, 8 and 9. But the present study did not find significant markers (P < 1.00E-05) in the A sub-genome, which is homologous to pearl millet chromosome 1 to 7. In general, a total of 67 SNPs were detected for all agronomic traits of which 47 were repeated across traits or treatment conditions (Table 9).

Forage quality of individual genotypes can be altered by abiotic factors such as season and soil moisture; hence, the assessment of plant performance and adaptability in different soil water conditions and seasons is important. A total of 50 SNPs were significantly associated with all nutritional traits, except OM (Appendix Table 2). One of the key nutritional traits in forage species is crude protein content (CP) and this study only identified 2 SNPs associated with CP content during the dry season in both soil moisture conditions. A study by Muktar *et al.* (2022) did not detect SNPs for CP content. Relatively, a higher number of SNPs were detected for ADF and ADL and these two traits were positively correlated (Figures 3). Relatively, a fewer number of SNPs were identified for NDF but a higher number for ADL. Since ADL and NDF significantly and positively correlated, SNPs identified for ADL can also be used for the selection of NDF traits.

Functional annotation of significant SNPs detected in the GWAS study showed interesting results. For example, SNP (SGWHAORA00000005_58573147) which was significant in both LL and TFW traits is positioned at the CpA0502823 gene in Napier grass. Blastx query of this gene, against NCBI database, showed significant similarity with FACT protein gene families. Several studies on this protein family revealed its role in the growth and

development of the vegetative and reproductive parts of the model and other species (Lolas *et al.*, 2010; Van Lijsebettens *et al.*, 2010).

Another SNP (SGWHAORA00000003_98130331) which was significantly associated with nutritional trait ADL is in the CpA0302294 gene in Napier grass and showed significant similarity with genes that regulate heat stress in rice (Nguyen et al 2015). The above two examples show that the SNPs identified in this study are of effect in other species as well. To date, more than 400 Napier grass accessions were sequenced in this project, and more polymorphisms and associations are being detected (not part of this thesis). Therefore, these genomic tools will play a key role in the future Napier grass breeding and its full domestication.

6. SUMMERY AND CONCLUSION

Napier grass is a fast-growing perennial grass native to Sub-Saharan Africa that is largely used as animal feed and found in tropical and subtropical areas across the world. The ILRI fodder genebank has a variety of genetic resources of Napier grass that have been collected and conserved, but little information is known about its diversity and important agronomic features. As an initial breeding effort, analyzing genetic and phenotypic diversity, defining crucial agronomic features, and identifying significant and acceptable molecular markers is a critical step forward in improving Napier grass germplasm to develop high yielding, quality (nutritional) and wider adopted cultivars. To speed breeding efforts on Napier grass, whole-genome sequencing (WGS) is required and were used in this study.

Despite the possibility of breeding genetic enhancement, many breeding programs in SSA have yet to implement genomics-based breeding strategies. This is due to the limited capacity of national institutes in acquiring genotypic data for the crop of interest and this challenge is worse in orphan forage crops like Napier grass. The present study revealed genetic diversity across a global collection of Napier grass accessions and this diversity was anchored to phenotypic and nutritional variability via an association mapping study. The activities initiated in this project will lead to public sharing of a genomic database and SNPs for design breeding in Napier grass for new cultivars, which will be made available to SSA farmers. The results from a present study will be key to initiating molecular marker-based breeding in Napier grass and fastening its further improvement effort. Ultimately, improved forages will play a key role in improving livestock performance in SSA and alleviate rampant protein malnutrition in the region.

Agronomic traits such as plant height (PH), leaf width (LW) and length (LL), total fresh weight (TFW) and total dry weight (TDW) and nutritional traits such as Acid Detergent fibre

(ADF), acid detergent lignin (ADL), Neutral detergent fibre (NDF), crude protein (CP), Metabolizable Energy (ME) and in-vitro organic matter digestibility (IVOMD) were among measured traits. Significant differences were observed and showed higher mean value under MWS conditions. Furthermore, a genome-wide association study (GWAS) identified more than 100 SNPs, significantly associated ($P < 1.00E-05$) with both agronomic and nutritional traits. The finding obtained in the present study will helps to enhance our understanding of complex agronomic and nutritional traits in Napier grass and these genomic tools will serve as a valuable resource in future breeding programs to select high yielding and drought-tolerant varieties of Napier grass, suited for different agroecological zones.

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8. APPENDIX

Appendix Table 1. Passport data for the list of Napier grass accessions included in this Study

No	Accessions	Origin	No	Accessions	Origin	No	Accessions	Origin
1	Add-*g*	S. Africa	37	CNPGL_93182*pg*	NA	73	ILRI_16800*pg*	Zimbabwe
3	BAGCE_100*pg*	Brazil	38	CNPGL_93322*g*	NA	74	ILRI_16801*pg*	Zimbabwe
4	BAGCE_16*g*	Brazil	39	CNPGL_93375*pg*	NA	75	ILRI_16802*pg*	Zimbabwe
5	BAGCE_17*pg*	Costa Rica	40	CNPGL_94072*g*	NA	76	ILRI_16803*pg*	Zimbabwe
2	BAGCE_1*g*	Colombia	41	CNPGL_94131*pg*	NA	77	ILRI_16804*pg*	Zimbabwe

6	BAGCE_22*g*	NA	42	CNPGL_96211*pg*	NA	78	ILRI_16805*pg*	USA
7	BAGCE_24*g*	NA	43	CNPGL_96231*pg*	NA	79	ILRI_16806*pg*	USA
8	BAGCE_25*g*	India	44	CNPGL_96241*g*	NA	80	ILRI_16807*pg*	USA
9	BAGCE_30*pg*	Brazil	45	CNPGL_96273*pg*	NA	81	ILRI_16808*pg*	USA
10	BAGCE_34*pg*	Brazil	46	G1*g*	NA	82	ILRI_16809*pg*	USA
11	BAGCE_53*pg*	Brazil	47	ILRI_1026*pg*	Burundi	83	ILRI_16810*pg*	USA
12	BAGCE_56*g*	Brazil	48	ILRI_14355*pg*	Burundi	84	ILRI_16811*pg*	USA

Appendix Table 1. Continued.

13	BAGCE_63*g*	Cuba	49	ILRI_14389*pg*	Ethiopia	85	ILRI_16812*pg*	USA
15	BAGCE_75*g*	Brazil	50	ILRI_14982*pg*	Nigeria	86	ILRI_16813*pg*	USA
14	BAGCE_7-*g*	Brazil	51	ILRI_14983-*pg*	USA	87	ILRI_16815-*pg*	USA
16	BAGCE_80-*g*	Brazil	52	ILRI_14984-*pg*	USA	88	ILRI_16816-*pg*	USA

17	BAGCE_81-*pg*	Brazil	53	ILRI_15357-*pg*	USA	89	ILRI_16817-*pg*	USA
18	BAGCE_86-*pg*	NA	54	ILRI_15743-*pg*	NA	90	ILRI_16818-*pg*	USA
19	BAGCE_93-*g*	NA	55	ILRI_16782-*pg*	Namibia	91	ILRI_16819-*pg*	USA
20	BAGCE_94-*g*	NA	56	ILRI_16783-*pg*	Tanzania	92	ILRI_16821-*pg*	USA
21	BAGCE_97-*pg*	NA	57	ILRI_16784-*pg*	Tanzania	93	ILRI_16822-*pg*	Zimbabwe
22	CNPGL_0011*-*pg*	NA	58	ILRI_16785-*pg*	Tanzania	94	ILRI_16834-*pg*	Malawi
23	CNPGL_91062-*g*	NA	59	ILRI_16786-*pg*	Tanzania	95	ILRI_16835-*pg*	Zimbabwe
24	CNPGL_91112-*g*	NA	60	ILRI_16787-*pg*	Swaziland	96	ILRI_16836-*pg*	Zimbabwe
25	CNPGL_91251-*g*	NA	61	ILRI_16788-*pg*	Swaziland	97	ILRI_16837-*pg*	Zimbabwe

26	CNPGL_921333-*pg*	NA	62	ILRI_16789-*pg*	Swaziland	98	ILRI_16838-*pg*	Zimbabwe

Appendix Table 1. Continued.

27	CNPGL_921901-*g*	NA	63	ILRI_16790-*pg*	Swaziland	99	ILRI_16839-*pg*	Zimbabwe
28	CNPGL_921987-*pg*	NA	64	ILRI_16791-*pg*	NA	100	ILRI_16840-*pg*	Zimbabwe
29	CNPGL_92382-*g*	NA	65	ILRI_16792-*pg*	Swaziland	101	ILRI_16902-*pg*	Zimbabwe
30	CNPGL_92562-*pg*	NA	66	ILRI_16793-*pg*	Mozambique	102	ILRI_18438-*pg*	Swaziland
31	CNPGL_92663-*pg*	NA	67	ILRI_16794-*p*g	Cuba	103	ILRI_18448-*pg*	Tanzania
32	CNPGL_92792-*pg*	NA	68	ILRI_16795-*pg*	Mozambique	104	ILRI_18662-*pg*	Tanzania

33	CNPGL_93011-*pg*	NA	69	ILRI_16796-*pg*	Zimbabwe	105	N19-*g*	USA
34	CNPGL_93042-*pg*	NA	70	ILRI_16797-*pg*	Zimbabwe	106	N228-*g*	USA
35	CNPGL_93061-*g*	NA	71	ILRI_16798-*pg*	Zimbabwe	107	N36-*g*	USA
36	CNPGL_93081-*g*	NA	72	ILRI_16799-*pg*	Zimbabwe	108	PION-*pg*	Brazil

Pg *refers to both phenotyped and genotyped accessions and* ***g*** *refers to only genotyped accessions by WGS,* NA=not available

Appendix Table 2. Passport Data of 84 Accessions of Napier Grass Used for the Study

	Accession code	Genus	Species	Origin	Collection	Colour
1	ILRI_1026	Pennisetum	purpureum	Burundi	ILRI	
2	ILRI_14355	Pennisetum	purpureum	Burundi	ILRI	
3	ILRI_14389	Pennisetum	purpureum	Ethiopia	ILRI	Green
4	ILRI_14982	Pennisetum	purpureum	Nigeria	ILRI	Green
5	ILRI_14983	Pennisetum	Purpureum x glaucum	USA	ILRI	Green
6	ILRI_14984	Pennisetum	purpureum	USA	ILRI	Green
7	ILRI_15357	Pennisetum	purpureum	USA	ILRI	Green
8	ILRI_15743	Pennisetum	purpureum x glaucum	NA	ILRI	Green
9	ILRI_16621	Pennisetum	purpureum	USA	ILRI	Green
10	ILRI_16782	Pennisetum	purpureum	Namibia	ILRI	Green
11	ILRI_16783	Pennisetum	purpureum	Tanzania	ILRI	Green
12	ILRI_16784	Pennisetum	purpureum	Tanzania	ILRI	Green
13	ILRI_16785	Pennisetum	purpureum	Tanzania	ILRI	Green

Appendix Table 2.continued

14	ILRI_16786	Pennisetum purpureum	Tanzania	ILRI	Green
15	ILRI_16787	Pennisetum purpureum	Swaziland	ILRI	Green
16	ILRI_16788	Pennisetum purpureum	Swaziland	ILRI	Green
17	ILRI_16789	Pennisetum purpureum	Swaziland	ILRI	Green
18	ILRI_16790	Pennisetum purpureum	Swaziland	ILRI	Green
19	ILRI_16791	Pennisetum purpureum	NA		Green
20	ILRI_16792	Pennisetum purpureum	Swaziland	ILRI	
21	ILRI_16793	Pennisetum purpureum	Mozambique	ILRI	Green
22	ILRI_16794	Pennisetum purpureum	Cuba	ILRI	Green
23	ILRI_16795	Pennisetum purpureum	Mozambique	ILRI	Green
24	ILRI_16796	Pennisetum purpureum	Zimbabwe	ILRI	Green
25	ILRI_16797	Pennisetum purpureum	Zimbabwe	ILRI	Green
26	ILRI_16798	Pennisetum purpureum	Zimbabwe	ILRI	Green
27	ILRI_16799	Pennisetum purpureum	Zimbabwe	ILRI	Green

Appendix Table 2.continued

28	ILRI_16800	Pennisetum purpureum	Zimbabwe	ILRI	Green
29	ILRI_16801	Pennisetum purpureum	Zimbabwe	ILRI	Green
30	ILRI_16802	Pennisetum purpureum	Zimbabwe	ILRI	Green
31	ILRI_16803	Pennisetum purpureum	Zimbabwe	ILRI	Green
32	ILRI_16804	Pennisetum purpureum	Zimbabwe	ILRI	Green
33	ILRI_16805	Pennisetum purpureum	USA	ILRI	Green
34	ILRI_16806	Pennisetum purpureum	USA	ILRI	Green
35	ILRI_16807	Pennisetum purpureum	USA	ILRI	Green
36	ILRI_16808	Pennisetum purpureum	USA	ILRI	Green
37	ILRI_16809	Pennisetum purpureum	USA	ILRI	Green
38	ILRI_16810	Pennisetum purpureum	USA	ILRI	Green
39	ILRI_16811	Pennisetum purpureum	USA	ILRI	Green
40	ILRI_16812	Pennisetum purpureum	USA	ILRI	Green
41	ILRI_16813	Pennisetum purpureum	USA	ILRI	Green

Appendix Table 2. continued

42	ILRI_16814	Pennisetum purpureum	USA	ILRI	Green
43	ILRI_16815	Pennisetum purpureum	USA	ILRI	Green
44	ILRI_16816	Pennisetum purpureum	USA	ILRI	Green
45	ILRI_16817	Pennisetum purpureum	USA	ILRI	Green
46	ILRI_16818	Pennisetum purpureum	USA	ILRI	Green
47	ILRI_16819	Pennisetum purpureum	USA	ILRI	Green
48	ILRI_16821	Pennisetum purpureum	USA	ILRI	Green
49	ILRI_16822	Pennisetum purpureum	Zimbabwe	ILRI	Green
50	ILRI_16834	Pennisetum purpureum	Malawi	ILRI	Green
51	ILRI_16835	Pennisetum purpureum x glaucum	Zimbabwe	ILRI	Green
52	ILRI_16836	Pennisetum purpureum x glaucum	Zimbabwe	ILRI	Green
53	ILRI_16837	Pennisetum purpureum	Zimbabwe	ILRI	Green
54	ILRI_16838	Pennisetum purpureum x glaucum	Zimbabwe	ILRI	Green
55	ILRI_16839	Pennisetum purpureum x glaucum	Zimbabwe	ILRI	Green

Appendix Table 2. continued

56	ILRI_16840	Pennisetum	purpureum	Zimbabwe	ILRI	Green
57	ILRI_16902	Pennisetum	Purpureum x glaucum	Zimbabwe	ILRI	Green
58	ILRI_17798	Pennisetum	purpureum	Zimbabwe	ILRI	Green
59	ILRI_18438	Pennisetum	purpureum	Swaziland	ILRI	Green
60	ILRI_18448	Pennisetum	purpureum	Tanzania	ILRI	Green
61	ILRI_18662	Pennisetum	purpureum	Tanzania	ILRI	Green
62	BAGCE-1	Pennisetum	Purpureum x glaucum	South_Africa	ILRI	Green
63	BAGCE-100	Pennisetum	purpureum	Colombia	EMBRAPA_collection	Green
64	BAGCE-16	Pennisetum	purpureum	Brazil	EMBRAPA_collection	Green
65	BAGCE-17	Pennisetum	purpureum	Brazil	EMBRAPA_collection	Green
66	BAGCE-22	Pennisetum	purpureum	Costa Rica	EMBRAPA_collection	Green
67	BAGCE-24	Pennisetum	purpureum	NA	EMBRAPA_collection	Green
68	BAGCE-25	Pennisetum	purpureum	NA	EMBRAPA_collection	Green
69	BAGCE-30	Pennisetum	purpureum	India	EMBRAPA_collection	Green

Appendix Table 2. continued

70	BAGCE-343	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
71	BAGCE-53	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
72	BAGCE-56	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
73	BAGCE-63	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
74	BAGCE-7	Pennisetum purpureum	Cuba	EMBRAPA_collection	Green
75	BAGCE-75	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
76	BAGCE-80	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
77	BAGCE-81	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
78	BAGCE-86	Pennisetum purpureum	Brazil	EMBRAPA_collection	Green
79	BAGCE-90	Pennisetum purpureum	NA	EMBRAPA_collection	Green
80	BAGCE-94	Pennisetum purpureum	NA	EMBRAPA_collection	Green
81	BAGCE-97	Pennisetum purpureum	NA	EMBRAPA_collection	Green
82	CNPGL_00-1-1	Pennisetum purpureum	NA	EMBRAPA_collection	Green
83	CNPGL_91-06-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple

84	CNPGL_91-11_-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
85	CNPGL_91-25-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
86	CNPGL_92-133-3	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
87	CNPGL_92-198-7	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple
88	CNPGL_92-190-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple
89	CNPGL_92-38-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
90	CNPGL_92-56-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
91	CNPGL_92-66-3	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
92	CNPGL_9279-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
93	CNPGL_93-01-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
94	CNPGL_93-04-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
95	CNPGL_93-06-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
96	CNPGL_93-08-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
97	CNPGL_93-18-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
98	CNPGL_93-32-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple

Appendix Table 2. continued

99	CNPGL_93_-37-5	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple
100	CNPGL_94-07-2	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
101	CNPGL_94-13-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
102	CNPGL_96-21-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
103	CNPGL_96-23-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple
104	CNPGL_96-24-1	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Purple
105	CNPGL_96-27-3	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green
106	PIONEIRO	Pennisetum purpureum	NA	EMBRAPA_elite_lines	Green

Appendix Table 3. Analysis of Variance for agronomic and nutritional Traits of Napier grass accessions under different moisture stress condition

	IVOMD				ME			
	Df	Sum Sq	Mean Sq	Pr(>F)	Df	Sum Sq	Mean Sq	Pr(>F)
Accessions	83.00	2196.16	26.4***	0.00	83.00	30.20	0.36***	0.00
Season (dry/wet)	1.00	6033.75	6033.75***	0.00	1.00	154.35	154.3***	0.00
Moisture Condition (sws/mws	1.00	2232.36	2232.3***	0.00	1.00	24.64	24.6***	0.00
Accessions:Season	83.00	851.65	10.26ns	0.46	83.00	12.63	0.15ns	0.99
Accession* Moisture Condition	83.00	652.27	7.8ns	0.94	83.00	12.93	0.15ns	0.99
Season:MC	1.00	1286.97	1286.9ns	0.00	1.00	16.42	16.4***	0.00
Accession*Season*MC	83.00	279.06	3.3ns	1.00	83.00	6.01	0.07ns	
Error	4208.00	42877.50	10.1		4207.00	982.38	0.2	
	CP				NDF			
Accessions	83.00	3804.91	45.8***	0.00	83.00	3348.09	40.3***	0.00

Appendix Table 3 continued…

Season (dry/wet)	1.00	115.73	115.7***	0.00	1.00	20606.77	20606.7**	0.00
Moisture Condition (sws/mws	1.00	3256.83	3256.8***	0.00	1.00	867.28	867.3***	0.00
Accessions:Season	83.00	1592.77	19.1***	0.00	83.00	1757.15	21.17***	0.00
Accession* Moisture Condition	83.00	902.78	10.87**	0.03	83.00	264.54	3.239ns	1.00
Season:Moisture Condition	1.00	1724.83	1724.8***	0.00	1.00	0.62	0.6ns	0.76
Accession*Season*MC	83.00	327.33	3.9ns	1.00	83.00	269.64	3.2ns	1.00
Error	4208.00	34570.44	8.2		4208.00	29301.24	6.96	
	ADL				ADF			
Accessions	83.00	68.81	0.8ns	0.06	83.00	3154.21	38***	0.00
Season (dry/wet)	1.00	1448.53	1448.5***	0.00	1.00	30643.29	30643.29***	0.00
Moisture Condition (sws/mws	1.00	8.98	8.9***	0.00	1.00	2752.77	2752.77***	0.00
Accessions:Season	83.00	22.16	0.26ns	1.00	83.00	1317.40	15.8***	0.01
Accession* Moisture Condition	83.00	5.33	0.06ns	1.00	83.00	592.07	7.1ns	0.99
Season:Moisture Condition	1.00	1.55	1.5ns	0.12	1.00	2060.44	2060.4***	0.00

Appendix Table 3 continued…

Accession*Season*MC	83.00	5.51	0.06ns	1.00	83.00	319.08	3.8ns	1.00
Error	4208.00	2762.29	0.6		4208.00	46625.12	11.08	
	OM				PH			
Accessions	83.00	2366.03	28.5***	0.00	83.00	134925.02	1625.6***	0.00
Season (dry/wet)	1.00	638.43	638.4***	0.00	1.00	2110141.83	2110141.8***	0.00
Moisture Condition (sws/mws	1.00	59.04	59.04***	0.00	1.00	33.30	33.3ns	0.75
Accessions:Season	83.00	1078.90	12.99***	0.00	83.00	95941.63	1155.9***	0.00
Accession*MC	83.00	263.95	3.1ns	0.45	83.00	237.45	2.86ns	1.00
Season:MC	1.00	51.77	51.8***	0.00	1.00	42.99	42.9ns	0.72
Accession*Season*MC	83.00	207.78	2.5ns	0.91	83.00	133.69	1.6ns	1.00
Error	4216.00	13249.58	3.1		4267.00	1441603.72	337.8	
	TFW				LL			
Accessions	83.00	400219.57	4821.9***	0.00	83.00	278288.68	3352.8***	0.00
Season (dry/wet)	1.00	1705197.10	1705197***	0.00	1.00	1279913.25	1279913.2***	0.00

Appendix Table 3 continued…

Moisture Condition (sws/mws	1.00	1.22	1.21ns	0.96	1.00	128.47	128.5ns	0.40
Accessions:Season	83.00	264854.16	3191***	0.00	83.00	28145.68	339.1ns	0.00
Accession* Moisture Condition	83.00	653.75	7.8ns	1.00	83.00	1339.95	16.1ns	1.00
Season:Moisture Condition	1.00	59.83	59.8ns	0.73	1.00	78.29	78.2ns	0.51
Accession*Season*MC	83.00	343.14	4.1ns	1.00	83.00	1475.49	17.8ns	1.00
Error	4267.00	2089077.72	489.5		3499.00	644195.95	184.1	
	LW				TN			
Accessions	83.00	36558.25	440.5***	0.00	83.00	6063399.00	73052***	0.00
Season (dry/wet)	1.00	67246.27	672.4***	0.00	1.00	5588928.14	5588928.1***	0.00
Moisture Condition (sws/mws	1.00	216.64	216.6***	0.00	1.00	9176.63	9176.69***	0.04
Accessions:Season	83.00	5306.88	63.9ns	0.00	83.00	1299656.19	15658.5ns	0.00
Accession* Moisture Condition	83.00	146.90	1.79ns	1.00	83.00	41832.40	504ns	1.00
Season:Moisture Condition	1.00	219.26	219.26***	0.00	1.00	197.56	197.55ns	0.76
Accession*Season*MC	83.00	129.51	1.56ns	1.00	83.00	38061.99	458.5ns	1.00

Appendix Table 3 continued…

Error	4267.00	40724.33	9.5		4267.00	8972798.87	2102.8
	TDW						
Accessions	83.00	22125.91	266.5***	0.00			
Season (dry/wet)	1.00	81826.29	81826.2***	0.00			
Moisture Condition (sws/mws	1.00	47.45	47.4ns	0.09			
Accessions:Season	83.00	14038.80	169.1***	0.00			
Accession* Moisture Condition	83.00	24.62	0.29ns	1.00			
Season:Moisture Condition	1.00	5.15	5.15ns	0.58			
Accession*Season*MC	83.00	15.60	0.18ns	1.00			
Error	4266.00	70884.13	16.6				

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), TN= tiller number (mm), TFW= total fresh weight (gr), TDW = total dry weight (g), NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter, MC=moisture condition, mws=moderate water stress condition, sws=severe water stress condition

Appendix Table 4. Combined Analysis of correlation among and between Agronomic and nutritional Traits

	TFW	PH	TDW	TN	LW	LL	NDF	ADF	ADL	OM	CP	Me	IOMD
TFW	1												
PH	0.78	1											
TDW	0.99	0.78	1										
TN	0.7	0.47	0.65	1									
LW	0.56	0.48	0.59	0.08	1								
LL	0.67	0.58	0.68	0.28	0.83	1							
NDF	-0.03	0.19	0.02	-0.26	0.04	-0.05	1						
ADF	0.33	0.32	0.36	0.08	0.44	0.41	0.41	1					
ADL	0.08	0.21	0.05	-0.02	-0.03	-0.03	0.4	0.5	1				
OM	-0.24	-0.04	-0.2	-0.39	-0.24	-0.27	0.76	-0.1	0.29	1			
CP	-0.1	-0.2	-0.15	0.1	-0.35	-0.31	-0.49	-0.69	-0.23	-0.08	1		
Me	0	-0.07	-0.03	0.08	-0.21	-0.17	-0.31	-0.73	-0.47	0.11	0.68	1	
IOMD	-0.06	-0.15	-0.09	0.09	-0.27	-0.26	-0.53	-0.76	-0.45	-0.11	0.89	0.87	1

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), TN= tiller number (mm), TFW= total fresh weight (gr), TDW = total dry weight (g), NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter, MC=moisture condition, mws=moderate water stress condition, sws=severe water stress condition

ppendix Table 5.

Appendix Table 5. Associated markers with Agronomic and Nutritional traits and its alleles those crossed the threshold levels of FarmCPU (P < 1.00E-05)

Traits	Season	Treatment	Chromosome	Position	P.value	maf	FDR_Adjusted _P-values	effect
				Phenotypic traits				
LL	Dry	MWS	A05	58573147	0.00	0.14	0.00	-0.37
			B02	40040366	0.00	0.38	0.00	0.16
			B07	3247644	0.00	0.48	0.00	0.52
			B06	47707055	0.00	0.44	0.00	0.42
			B05	17150204	0.00	0.07	0.00	0.26
			B06	105448364	0.00	0.04	0.00	-0.33
			B04	67364239	0.00	0.08	0.01	0.20
			A06	56903089	0.00	0.13	0.02	-0.11
			B04	65799675	0.00	0.04	0.02	0.26

Appendix Table 5 continued..

		SWS	A07	61387737	0.00	0.05	0.00	-0.47
			A03	57635505	0.00	0.09	0.00	-0.23
			B01	93985305	0.00	0.03	0.00	-0.34
			A06	52372976	0.00	0.03	0.01	0.33
			A06	26390891	0.00	0.11	0.01	0.18
			A01	72141340	0.00	0.04	0.01	0.26
			B02	129380546	0.00	0.09	0.02	0.18
	Wet	MWS	A05	58573147	0.00	0.14	0.00	-0.37
			B02	40040366	0.00	0.38	0.00	0.16
			B07	3247644	0.00	0.48	0.00	0.52
			B06	47707055	0.00	0.44	0.00	0.42
			B05	17150204	0.00	0.07	0.00	0.26
			B06	105448364	0.00	0.04	0.00	-0.33
			B04	67364239	0.00	0.08	0.01	0.20

Appendix Table 5 continued..

				A06	56903089	0.00	0.13	0.02	-0.11
				B04	65799675	0.00	0.04	0.02	0.26
			SWS	B03	23465232	0.00	0.49	0.00	-1.91
				A07	3335682	0.00	0.04	0.00	-0.34
				B02	135185230	0.00	0.13	0.01	0.14
				A05	58573147	0.00	0.14	0.01	-0.23
				A01	37905978	0.00	0.05	0.06	-0.26
PH		Wet	MWS	B03	48939934	0.00	0.17	0.00	0.35
				A01	92750473	0.00	0.08	0.00	-0.39
				B06	45734009	0.00	0.08	0.00	-0.47
				B01	156728589	0.00	0.26	0.00	-0.31
				A03	45421871	0.00	0.25	0.01	0.25
				A05	76791930	0.00	0.09	0.02	0.27
				B07	37004642	0.00	0.24	0.03	-0.16

Appendix Table 5 continued…

			B01	91923462	0.00	0.06	0.05	-0.32
		SWS	B06	45734009	0.00	0.08	0.00	-0.67
			B01	156728589	0.00	0.26	0.00	-0.33
			A04	82188016	0.00	0.23	0.00	0.25
			B03	48939934	0.00	0.17	0.00	0.25
			A01	92750473	0.00	0.08	0.03	-0.25
			B02	104421462	0.00	0.10	0.03	-0.24
			A04	63562253	0.00	0.03	0.03	-0.40
			B07	37004642	0.00	0.24	0.03	-0.17
			B02	109877092	0.00	0.47	0.03	-0.37
TDW	DRY	MWS	A04	112701449	0.00	0.11	0.15	-0.24
			A01	26467349	0.00	0.07	0.15	-0.28
	WET	MWS	A04	59427440	0.00	0.03	0.22	-1.19
		SWS	A04	59427440	0.00	0.03	0.18	-1.19

Appendix Table 5 continued…

TFW	Dry	MWS	A04	112701449	0.00	0.11	0.06	-0.48
			A01	26467349	0.00	0.07	0.06	-0.57
	Wet	MWS	A05	58573147	0.00	0.14	0.00	-0.54
			B04	57738396	0.00	0.06	0.00	-0.83
			A04	59427440	0.00	0.03	0.00	-1.21
			B06	105448364	0.00	0.04	0.02	-0.83
			B07	3891037	0.00	0.10	0.06	-0.46
			A04	39159991	0.00	0.13	0.06	0.36
		SWS	A05	58573147	0.00	0.14	0.00	-0.67
			B04	57738396	0.00	0.06	0.00	-1.10
			A04	59427440	0.00	0.03	0.00	-1.16
			B07	27574582	0.00	0.09	0.00	0.50
			B06	105448364	0.00	0.04	0.01	-0.71
			B07	16460222	0.00	0.04	0.02	-0.62

Appendix Table 5 continued..

			A04	40544478	0.00	0.06	0.03	-0.55
TN	Wet	MWS	B01	140419403	0.00	0.07	0.21	-1.74
				Nutritional traits				
ADL	Dry	MWS	B06	57938152	0.00	0.04	0.00	-0.07
			B05	72665475	0.00	0.24	0.00	-0.01
			B03	1483706	0.00	0.18	0.01	-0.01
		SWS	B04	8598020	0.00	0.08	0.00	-0.03
			B04	106867472	0.00	0.11	0.00	-0.03
			B02	37672601	0.00	0.18	0.00	0.02
			A06	72514270	0.00	0.10	0.01	0.02
			B01	42562394	0.00	0.44	0.02	-0.01
			A01	77087943	0.00	0.08	0.03	-0.02
	Wet	MWS	A05	55011882	0.00	0.34	0.00	0.03
			B04	44156178	0.00	0.19	0.00	0.03

Appendix Table 5 continued..

				B06	38504570	0.00	0.05	0.00	0.04
				A04	54429733	0.00	0.08	0.00	-0.02
				A03	40755983	0.00	0.10	0.00	-0.02
				A03	98130331	0.00	0.28	0.00	0.01
				B06	63946058	0.00	0.14	0.00	-0.02
				B02	72339600	0.00	0.48	0.02	-0.02
			SWS	A03	98130331	0.00	0.28	0.18	0.03
CP		Dry	MWS	A04	39328212	0.00	0.38	0.27	-0.19
			SWS	A02	153925499	0.00	0.05	0.16	0.28
			SWS	B06	36372759	0.00	0.40	0.00	0.07
				B01	14395204	0.00	0.47	0.00	-0.03
				B01	58346445	0.00	0.10	0.00	0.05
				B02	107468105	0.00	0.05	0.00	0.06
				A04	101408617	0.00	0.06	0.00	0.04

Appendix Table 5 continued..

			B02	93527062	0.00	0.15	0.01	-0.02
			B04	81738561	0.00	0.05	0.01	0.05
			A05	122242907	0.00	0.06	0.02	0.05
			A04	62759479	0.00	0.15	0.03	0.02
			B01	155974349	0.00	0.37	0.03	-0.03
			B05	66722193	0.00	0.15	0.03	0.02
ME	Dry	SWS	A02	153925499	0.00	0.05	0.00	0.04
			B06	36372759	0.00	0.40	0.00	0.02
			A02	43421441	0.00	0.37	0.00	-0.01
			B05	38697773	0.00	0.50	0.01	-0.05
			B02	93527062	0.00	0.15	0.01	-0.01
			B02	110718947	0.00	0.04	0.01	0.02
			A04	62759479	0.00	0.15	0.05	0.01
NDF	Wet	MWS	A06	52372976	0.00	0.03	0.22	0.14
		SWS	A06	52372976	0.00	0.03	0.22	0.14
	Wet	MWS	A03	30046864	0.00	0.38	0.28	0.02
		SWS	A03	62335723	0.00	0.24	0.15	0.03
			B06	73559745	0.00	0.15	0.15	0.04

PH=plant height (cm), (LL= leaf length) (mm), LW= leaf width (mm), TN= tiller number (mm), TFW= total fresh weight (gr), TDW = total dry weight (g), NDF =neutral detergent fibre, ADF= Acid detergent fibre, ADL =acid detergent lignin, CP =crude protein, IVOMD =in vitro organic matter digestibility, Me =metabolizable energy, OM =organic matter, MC=moisture condition, mws=moderate water stress condition, sws=severe water stress condition

BIOGRAPHICAL SKETCH

A study was conducted by Hailu Lire Wachamo, who was born in South Ethiopia Hadia Zone Soro District Akama kebele. He attended elementary education at Akama primary School from 1998-2004 G.C and secondary and preparatory school at Gimbichu Secondary and preparatory School from 2005-2009 G.C and then After I was joined Wolaita Sodo University in 2010 and graduated with the Bachelor of Science degree in Plant Sciences on July 12, 2012. I was employed by the Southern Nation Nationalities peoples rural job opportunity Creation, and development Agency soro Agricultural office rural job opportunity creation and development cluster Expert from January 8, 2013- April 30, 2014. Then, from 2014-2018 Researcher in the Ethiopian Institute of Agricultural Research (EIAR) wondogenet Agricultural Center. Then he was joined Hawassa University to pursue a Master of Science Degree in Plant Biotechnology and, now he is to report the thesis research results.