ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

Molecular Genetic Diversity Assessment of Finger Millet (*Eleusine coracana* (L.) Gaertn) with different Seed Coat Color Using Inter Simple Sequence Repeat (ISSR) Marker

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A Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)

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January, 2018

Addis Ababa University

Acknowledgements

First of all I would like to express my deepest gratitude to my advisors, Dr. Kassahun Tesfaye, for his valuable guidance, sustained encouragement and immense inspiration throughout the course of my research study and who gave me an opportunity to work in his lab, as well as Dr. Teklehaimanot Haileslassie, for his meticulous guidance, constructive criticism, and for providing excellent research facilities throughout the period of this investigation and during the writing up of this thesis.

I would like to show my gratitude to Ms. Hawi Nugussie, a PhD candidate, for her advice and support during the lab work of this study. The discussions of technical and interpretational problems were extremely helpful. I need to thank Eden, for helping me with the marker analysis, and statistical analysis.

Big thank goes to my family who believed in me and let me make my own identity by stepping out of home. I am grateful to their prayers, love and support.

Last but not the least, I need to thank my friends who have made me feel at home in Addis Ababa. There are too many of you to name here, but Biru thanks for your continued support and all the goodies throughout my program. I appreciate the immense support of my friends. Worthy of special mention are Guesh Mulaw and Baraki Haile, for the constant reminder and helped me in every possible way to make my efforts. To everyone else who has assisted me in any way, great or small, may God bless you abundantly!

I am indebted to Aksum University for giving me the opportunity to join the graduate program and provision of financial support. I would also thank Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, for accepting me as a graduate student and provision of all facilities and resources during this study. I would also like to take this opportunity to thank the Staff Members of the Department for sharing their knowledge and skills without any reservation. I would also thank DAAD for the financial support. My thanks also go to the Farmers of Woredas of Ahiforom, Mereb-leke, Asgede-tsimbla, and Laelay-adyabo for providing me seeds of finger millet (*Eleusine coracana)* accessions.

LIST OF ABBREVIATIONS

2D	Two dimensions
AMOVA	Analysis of Molecular Variance Averages
bp	Base pair
cDNA	Complimentary DNA
CSA	Central Statistical Authority
CTAB	Cetyl Tri-methyl Ammonium Bromide
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agricultural Organization
H	Nei"s Genetic diversity
I	Shannon information index
ICRISAT	International Crops Research Institute for Semi-Arid Tropics
ISSR	Inter Simple Sequence Repeat
NJ	Neighbor Joining
TBE buffer	Tris-borate-EDTA buffer
TE buffer	Tris-EDTA buffer
UPGMA	Un-weighted Pair Group Method using Arithmetic

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ABSTRACT

Finger millet (Eleusine coracana (L.) Gaertn.), is a staple crop with high nutritional profile cultivated mainly in developing countries and widely cultivated in Ethiopia. It is an allotetraploid (2n= 4x= 36 chromosomes), that belongs to the Poaceae family. It is characterized by its ability to grow well on a variety of environmental conditions, its excellent grain storage quality and the ability to withstand significant levels of salinity. Scientific research aimed at improving this important cereal has been negligible and it is regarded as one of the orphaned crop species. The aim of this study was to determine the genetic variability and patterns of diversity of finger millet (Eleusine coracana) with varying seed coat color. The study was done on 72 finger millet accessions with variable seed coat color collected from two zones of Tigray region for molecular characterization. Molecular characterization of the total number of bands, average polymorphic bands, average percent polymorphism, and average expected gene diversity (Hi) generated from 6 ISSR markers used in the study. The markers generated 45 loci with 30 polymorphic and 15 monomorphic loci, at an average of 7.5 loci per primer. The gene diversity (h) indexes of the four populations with varying seed coat color were calculated and the values ranged from 0.26 for Ahiferom, Mereb-Leke, and Asgede-tsimbla to 0.27 to Laelay-adyabo in according to their geographical location. Besides this, when the finger millet populations were grouped in to seed coat color (black, red, and white), the gene diversity (h) was 0.27 in black seed coat color and 0.26 each in red and white colors. The UPGMA cluster analysis revealed that the three varieties were grouped in to three major clusters at the demarcation of 73% genetic similarity. Cluster I comprised black and red seed coat color varieties from Ahiforom population and cluster II comprised three intermixed varieties from all population, and cluster III consisted of black and red seed coat colors from Mereb-leke population. The genotypes, Ahiforom population and Mereb-leke population showed the lowest similarity. Based on AMOVA, variation within population was higher (98%) as compared to among populations (2%). The higher dissimilarity between the Ahiforom and Mereb-leke genotypes makes them better candidates for inclusion in crop improvement programmes. Since,

hybrid vigor has a positive relation with genetic distance; hybridization between these two lines will provide progenies with greater variation at molecular level for selection of agronomically important traits.

Keywords: accession, *Eleusine coracana*, finger millet, diversity, ISSR, seed coat color

Table of contents Page

List of figures **Page**

List of tables **pages**

1. INTRODUCTION

1.1. Background of the Study

The word "millet" was derived from the Greek word “meline” and represents a diverse group of cereal crops that typically produce small seeds and is used loosely to refer to several types of small-seeded annual grasses (FAO and ICRISAT, 1996). Finger millet and its wild relatives are members of *Chloridoidea*, one of the primary subfamilies of the grasses, and belong to family Poaceae. It is said to have derived its name ‘*Eleusine’* from where cereals are worshipped. Its specific name *coracana* comes from its Ceylonese name ‘Kurakkan’.

Finger millet is an important cereal in Eastern Africa and is also widely cultivated in Southern India (Dida *et al*., 2008).). East Africa is recently reported as the center of origin and diversity of finger millet (FAO, 1998). It was domesticated since 5000 BC in eastern Africa possibly Ethiopia (Hilu et al., 1979). Some of its vernacular names in the countries where it is grown, include: Dagussa (Ethiopia), Wimbi (Swahili), Bulo (Uganda), Tellebun (Sudan) and Ragi (India) (Reddy *et al*., 2011). Its value stems from its versatility as a staple food, and its excellent grain storage quality attributed to its high polyphenol content and small grain size that prevent storage pests (Chetan and Malleshi, 2007). The fact that it grows on a variety of environmental conditions, particularly in marginal areas, makes it an ideal crop food security. Finger millet seed can lie dormant for weeks in times of drought, germinating at the onset of rains and can be harvested in just forty-five days (Styslinger, 2011). It can also withstand significant levels of salinity and is not overwhelmed by many serious diseases, except finger millet blast disease (National Research Council, 1996; Dida *et al*., 2007). Due to its high nutritional value, it is an important food security crop (Dida *et al*., 2007). Its calcium content is higher than that of other major cereals. It is also rich in iron as well as the amino acids leucine, tryptophan, phenylalanine, and methionine. Consequently, it is a major preventive agent against malnutrition (Barbeu, 1993; National Research Council, 1996). After grain harvest, the finger millet Stover (the leaves and stalks that remain after the grains are harvested) is an important source of fodder for livestock. This contributes to making finger millet of significant importance in food and feed security in areas that rely on crop and livestock farming (Krishnappa *et al*., 2009).

Ethiopia is a diverse country in terms of altitude, temperature, rainfall and soils types. Such diversity of environmental elements have been the cause for the existence of diverse vegetation, crop species and native varieties of crops that are observed in farmers' fields in most part of the country (Vavilov, 1951). According to Vavilov and other scientists, Ethiopia represents one of the major centers of genetic diversity. Vavilov (1951) indicated that some thirty species are connected with Ethiopia as a primary or secondary gene center. Eleven crops including finger millet (*Eleusine coracana*) were indicated as having their center of diversity in Ethiopia and local domesticates are known to have a wide genetic diversity. In Ethiopia, finger millet utilization is deep-rooted in the culture of the people. The grain is used for making the native bread, Injera, porridge, cake, soup, traditional breakfast called "chachabsa", malt, local beer and distilled spirit (Areki) alone or in mixture with maize, and barley. The straw is used for animal feed. The great merit of finger millet is that it can be stored for the period of up to ten years or more without deterioration and weevil damage (Dagnachew *et al*., 2014).

Production statistics show that 94% of global millet output is from developing countries mainly in Asia and Africa. In these countries, 95% of millet produced is consumed as food with a large number of households aiming to produce just enough to feed them. However, many often are unable to meet this goal (FAO and ICRISAT, 1996). Finger millet, which is high in energy, rich in micronutrients and essential amino acids can play a role in malnutrition and in achieving food security. Although finger millet has been grown as a subsistence crop for many decades, there has been very little scientific intervention aimed at improving and developing this important cereal (Kumari and Pande, 2010). This may be due to the fact that millets, in general, are of little economic importance to developed countries in comparison to major cereals such as maize, wheat, and rice (Kothari *et al*., 2005). Thus, finger millet is regarded as one of the orphaned crop species, which are crops with underexploited potential for contributing to food security, health (nutrition/medicinal), income generation and environmental services. This may partly be attributed to the fact that their importance is largely local or regional in scale (Jaenicke and Hoschle-Zeledon, 2006).

Finger millet took up about 456,171.71 hectares (4.46%), of the grain crop area. As to production, cereals contributed about 253,847,239.63 quintals (87.42%) of the grain production, and Finger millet made up 10,170,592.71 quintals (4%), of the grain production, in Ethiopia

(CSA, 2016). Finger millet accounts 11.51% of the cropland allotted to cereals in the Region, and 68.91%, 13.09%, of the cropland is allotted to cereals in Northwest, and central Zones of Tigray, respectively (CSA 2016).

Currently, the government of Ethiopia is working to ensure food security in the country through the increment of agricultural productivity and development of a green environment in combating global warming. To this end, understanding the genetic variability that exists in our staple crops such as finger millet is useful to enhance production and productivity. Genetic diversity is normally assessed by common morphological and biochemical traits. However, such traits are affected by such factors as the environment, development stage of the plant and the type of plant material, and also it requires several replications to establish the genotypic contributions. Hence, there is a need to look for a highly reliable and precise method for assessment of genetic variability with no environmental effects. Assessment of genetic diversity with molecular markers overcomes this problem. The use of molecular markers allows the direct assessment of genotypic variation at the DNA level. Marker analysis helps to understand the genetic makeup of the accessions and also make it possible to analyze the global organization of genetic diversity within a species, but comprehensive studies on finger millet diversity using morphological or molecular markers are generally limited (Bezaweletaw *et al.*, 2006).

So far, very few accessions have been characterized at the morphological level in Ethiopia (Daba *et al*., 2008, Wolie *et al*., 2011, Bezawuletaw *et al*., 2006, Tsehaye and Kebebew, 2002). To date only few efforts have been made to assess the genetic diversity among finger millet genotypes from various regions of the country. The objective of this study is, therefore, to determine the genetic variability and patterns of molecular level genetic diversity of finger millet accessions (*Eleusine coracana*) with varying seed coat colors namely, red, white and black, collected from two zones of Tigray assessed with the ISSR marker.

1.2. Statement of the Problem

Finger millet is a crop of immense importance in areas where it is grown. Its properties such as excellent grain storage quality, high nutritional value and ability to thrive in marginal areas make it an important food security crop. However, it still remains an orphaned crop with little research having been undertaken to improve it, as compared to the major cereals, such as maize, wheat, and rice. Yet, very few efforts have been made to assess the genetic diversity among finger millet accessions from Ethiopia at a molecular level. And there is little information on the extent of genetic diversity among finger millet accessions adapted to various agro-ecological areas of Ethiopia, and the scantly information available so far have not been linked to the extent of variation at molecular level among the three landraces finger millet accession with variable seed coat color.

1.3. Significance of the Study

Finger millet (*E. coracana*) is an important cereal crop in Ethiopia. Due to its high nutritional contents and excellent storage quality; it serves as a supplement for the main cereal crops. It is also a good source of micronutrients, such as calcium, zinc, potassium, etc. (Shashi *et al*., 2007). Its wide adaptability to diverse environments and cultural practices makes it a potential food crop. In spite of its importance, its productivity is relatively low in Ethiopia. Its low productivity has, amongst others, been due mainly to the low genetic yield potential of the types under cultivation coupled with traditional cultural practices, and other biotic and abiotic stresses. There is limited knowledge regarding the molecular genetic diversity in finger millet, limiting the efficiency of breeding programs. This, in turn, calls for systematic collection, characterization and evaluation, and conservation of the available finger millet (accession) genetic resources.

This study contributes in the choice of the best yield attributes either for selection or hybridization. Good amount of variability has been reported in finger millet crop for the various characters, such as height, flowering, maturity, and disease reaction, but it has not been fully exploited in breeding programs (Dagnachew *et al.*, 2014). It is, therefore, felt necessary to study the genetic variability at molecular level using ISSR markers in different accessions. This study will also have significance in increasing the available DNA sequence information and expand genetic marker resources for finger millet, and provide basic understanding of the genetic

variations in Ethiopian finger millet to serve in germplasm conservation, and other genomic research for future marker assisted breeding of the crop.

2. LITERATURE REVIEW

2.1. Origin, and Distribution of Finger Millet

Finger millet is believed to have originated in Ethiopia about 5000 years ago with domestication extending from the Ethiopian highlands to western Uganda (Dida and Devos 2006). Soon after, the crop was introduced into India through the sea trade that existed, whereupon the subcontinent became its secondary center of diversity (Dida et al., 2008; Liu and Peterson et al., 2011). It is also cultivated in Burma, southern parts of Tibet, Nepal, Malaysia, Sumatra, Sri Lanka, Philippines, Japan, China, Java, Iran, Afghanistan and in the Arabian peninsula (Bisht and Mukai 2002).

Most of the species are confined to Africa; however, *E. coracana* has made its way through Africa and the Indian subcontinent. *Eleusine* species occupy diverse habitats, ranging from open, dry places to forests under-covers. Finger millet is extensively grown in the semi-arid regions of Africa and India through the selection of genotypes adapted to the different agro ecological conditions (Werth et al., 1994; Das et al., 2007).

Besides, the crop is cultivated in diverse eco-geographical areas where it displays high variability in vegetative, floral, and seed morphology. Hilu and de Wet (1976) and Upadhyaya et al. (2007) identified three eco- geographical races: African highland race cultivated in the East African highlands, a lowland race grown in the lowlands of Africa and south Indian and an Indian race with its center of diversity in northeast India. The African highland race is the most primitive, diverse and the precursor of the lowland race, which was subsequently introduced to southern India that developed into a secondary center of diversity, resulting in the Indian race. Natural selection may have played a significant role in finger millet evolution (Hilu and de Wet, 1995a).

Archaeological evidence also indicates that finger millet was a staple crop of Southern Africa before the introduction of maize. Today, finger millet is cultivated in the Eastern and Southern Africa and is a major cereal in the northern, southern, and western parts of Ethiopia, displaying a lot of trait variability however not collected, characterized, and underutilized for breeding programmes (Dagnachew *et al.*, 2014).

2.2. Taxonomy

Based on inflorescence compactness and shape, finger millet is classified into races and subraces. Species *E. coracana* consists of two subspecies *africana* and *coracana*. Subspecies *africana* consists of two wild races *africana* and *spontanea*; and subspecies *coracana* consists of four cultivated races namely *elongata, plana, compacta*, and *vulgaris*. Each of these races is further classified into subraces. The race *elongata* has three subraces; *laxa, reclusa* and *sparsa*; race *plana* also consists of three subraces; *seriata, confundere* and *grandigluma*; race *compacta* has no subrace, while the race *vulgaris* has four subraces, *liliacea, stellata, incurvata* and *digitata* (Prasada Rao et al., 1993).

The genus *Eleusine* Gaertn. is a member of the tribe Chlorideae and comprises eight species, among which the cultivated *E. coracana* subsp. *coracana* (finger millet) is the most important subsistence crop in Africa and India in contrast to *E. indica* (goose grass), which is categorized as one of the most problematic weeds in the world (Holm *et al.*, 1977; Phillips, 1995; Devarumath *et al*., 2005). The genus is characterized by three different basic chromosome numbers of x = 8, 9 or 10; *E. intermedia, E. indica, E. floccifolia* and *E. tristachya* are diploids with 2n = 2x = 18; *E. multiflora* is a diploid with 2n = 2x = 16 and *E. jaegeri* is also diploid with 2x = 2n = 20 (Devarumath *et al*., 2005). The tetraploid *Eleusine spp.* comprise *E. coracana* subsp *coracana* and subsp. *africana* with 2n = 4x = 36 and *E. kigeziensis* with 2n = 38, which all probably have allopolyploid origins involving two diploid species with x = 9 or 10 (Bisht and Mukai, 2002; Neves, 2011). The most common vernacular names for finger millet include: finger, birdsfoot, African millet or ragi millet (in English); ragi, nagli, mandua or kurukkan (India); Dagussa (Ethiopia); Wimbi (in East Africa such as Kenya, Uganda and Tanzania); and *coracan* (French).

2.3. Cytology and Genome Donors of Finger Millet

Finger millet is an allopolyploid believed to have arisen in the highlands of eastern Africa through spontaneous hybridization of *Eleusine indica* (now a major weed) and another species of the same genus (Hilu, De Wet et al. 1979; Hilu and Johnson 1992; Liu, Triplett et al. 2011). It has been established that the A genome donor is the diploid, *E. indica* (Dida and Devos 2006). However, the identity of the B genome donor is still a subject of some debate. Based on chromosome numbers, three species, *E. floccifolia, E. tristachya* and *E. intermedia*, are

considered to be the most probable B genome donors to *E. coracana*, as they have the appropriate chromosome number of 2n=2x=18. Based on several crosses, and *in situ* chromosome hybridizations, it appeared that *E. floccifolia* is the most likely B genome donor (Bisht and Mukai 2002).

However, phylogeny studies grouped *E. coracana* subsp. *coracana* and *africana, E. indica,* and *E. kigeziensis* in one clade with *E. tristachya* as its sister group (Neves, Swire-Clark et al. 2005). The use of internal transcribed spacers (ITS) sequences and plastid sequences identified *E. indica* as the A genome donor for *Eleusine coracana,* but refuted the claim that *E. floccifolia* is its B genome donor. Another study supported the independent allotetraploid origin for *E. kigeziensis* and *E. coracana* subsp. *coracana* and *africana* clade by identifying diploids: *E. indica* and *E. tristachya* as maternal parents (Liu, Triplett *et al.* 2011) and indicated that the paternal parents for allotetraploid *E. coracana* may be extinct.

2.3.1. Genome Size

Finger millet is a tetraploid with a genome constitution of AABB, and base chromosome number of 9 (2n = 4x = 36). In 1991, the amounts of nuclear DNA for 30 collections belonging to 10 species of *Eleusine* were determined (Hiremath and Salimath 1991). Congeners of *E. coracana*, showed a 1C DNA content range from 2.55 pg in *E. africana* to 3.08 pg in *E. floccifolia* (Bennett 2012). Later in 1995, the genome size of the tetraploid crop was estimated to be 2.90pg by Feulgen microdensitometer (Mysore and Baird 1997). Most recently, the finger millet genome size has been measured as 1.8 pg/1C nucleus by flow cytometry (Dida, Ramakrishnan *et al*. 2007), corresponding to approximately 2509 Mbp (Bennett and Leitch 1995). This is larger than the sequenced 430 Mbp rice (*Oryza sativa*) genome (Pennisi 2007) but smaller than the 17000 Mbp wheat (*Triticum aestivum*) genome (Brenchley, and Spannagl *et al.*, 2012).

2.4. Reproductive Biology of Finger Millet

According to Ayyangar (1932) the complete emergence of the inflorescence requires about 10 days and flowering extends over at least 7 or 8 days. The plant flowers under a photoperiod of 11 hour and 30 minutes to 11 hours and 50 minutes (Kenneth and LeRoy, 1977). Finger millet with 9 compact and semi compact ear type undergo anthesis between 2 and 5 am, while panicles with

open spikes tend to flower between 1 and 3 am (Kenneth and LeRoy, 1977). The general tendency of the flowers is to open and progress from the top to the bottom in a finger. In a spikelet, however, the order is reversed and proceeds from the bottom to the top and from the bigger to the smaller flowers (Kenneth and LeRoy, 1977). The stigma remains receptive for a very short period after its emergence from glumes (Kenneth and LeRoy, 1977). The period of anthesis in the flower is very short and is conductive to self-pollination, but occasionally cross-pollination occurs (Kenneth and LeRoy, 1977).

2.5. Finger millet research in Ethiopia

Research on finger millet started in the early nineteenth century at Debrezeit Agricultural Research Center. It was merged with sorghum research at Melkassa Agricultural Research Center in 1986. About 13 improved varieties were released from the different research centers. However there are several gaps in terms of addressing peoples' preference on the type of varieties developed so far. For instance, in Metekel area, the two released varieties, Baruda and Dibatsi are red colored which are preferred for making local drinks termed *borde* by the Gumuz people. Productivity of white and black seeded finger millet varieties studied so far in the area had lower yield and were susceptible to lodging. Purple–black seed color is highly preferred in Northern Ethiopia due to the fact that farmers linked this type of finger millet to high yield, tolerant to moisture stress and poor soil fertility. But no variety was released in the area so far. White seeded finger millet is highly preferred for injera making in the several finger millet growing regions. But there are several yield and agronomic drawbacks associated with such varieties such as low yield, susceptibility to lodging and susceptibility to blast disease. Necho is the only white seeded released variety, which is adapted to higher altitude areas around Yilmana Densa district and fails to adapt in Western Ethiopia where the blast disease is a priority constraints. There has been very limited research on crop management and protection practices for finger millet. The agronomic recommendations used were variable across regions depending on the soil history and other environmental factors (Dagnachew *et al.*, 2014)

Yet very few studies have been made to assess the genetic diversity of finger millet accessions adapted to different agro-ecological areas of Ethiopia at molecular level some of these including: molecular level diversity using RAPD markers (Bezawuletaw, 2011); Assessment of genetic diversity, genotype by environment interaction, blast (*magnaporthe oryzae*) disease resistance,

and marker development for finger millet germplasm (Dagnachew *et al*., 2014); and Hydroponic Screening of Aluminium Tolerance and Genetic Diversity Analysis of Ethiopian finger millet (*Eleusine coracana* (L.) Gaertn) Genotypes as Revealed by Inter-Simple Sequence Repeat (ISSR) Markers (Haftom and Kassahun, 2015).

2.6. Uses of Finger Millet

Finger millet is principally grown by small-holder farmers and is often regarded as a poor man's food. It is the second most important cereal after maize in Uganda (Oduori, 2005). In Africa, finger millet is traditionally consumed in the form of porridge and bread flour, mainly for its flavor and aroma. Sprouted seeds (malted finger millet) are nutritious and easily digestible, and are hence recommended for infants and elderly (Oduori, 2005). In Zimbabwe, finger millet is consumed to satisfy traditional requirements or as nutritional supplements. Expectant or lactating mothers, babies and the sick are fed with these supplements. Finger millet finds its place in the beverage industry as well, where it is used to make traditional beer. As fodder, finger millet straw contains up to 61% total digestible nutrients (Oduori, 2005). Being rich in nutrients and minerals, the finger millet straw is reported to be more nutritious than that of pearl millet, wheat and sorghum (Bisht and Mukai, 2002).

Finger millet has a better nutrient profile than rice, corn or sorghum in terms of protein, fat, minerals and essential amino acids. The main storage protein (elusinin), contains good amounts of tryptophan, cysteine, methionine, and total aromatic amino acids, which are all crucial to human health and growth, but are deficient in most major crops. Because of such attributes, finger millet has been promoted as part of a solution to malnutrition, especially in parts of India (Singh and Raghuvanshi, 2012). Essential amino acids namely, tryptophan, threonine, lysine, methionine, valine and isoleucine were found to be present in great proportions in finger millet (FAO, 1991). The crop is also reported to have hypoglycemic, hypocholestrolemic and anti-ulcerative properties (Panwar *et al*., 2010).

Table 1: Nutritional profile of finger millet (Source; Singh and Raghuvanshi, 2012).

Composition of finger millet	Percentage
Carbohydrate	72-79.5%
Dietary fiber	12%
Protein	7%
Crude fat	1.3-1.8%
Ash content	1.7-4.13

2.7. Overview of Finger Millet Production in Ethiopia

Ethiopia is recognized as an important source of the public goods associated with crop genetic diversity conservation, as it is a primary or secondary center of diversity for several crops. The tremendous variation in altitude, temperature, rainfall, soil type and ecological setting, as well as the diverse social and cultural condition together with different levels of market integration are some of the possible explanations for the existence of remarkable genetic variation of crop varieties in the country (Cavatassi *et al*., 2006). Eleven crops including finger millet were indicated as having their center of diversity in Ethiopia (Kebere *et al*., 2007).

According to the CSA 2016/17, Meher Season Post-harvest Crop Production Survey indicated that a total land area of about 12,574,107.33 hectares was covered by grain crops i.e. cereals, pulses, and oilseeds, from which a total volume of about 290,385,593.21 quintals of grains was obtained, from private peasant holdings. Within the category of grain crops**,** cereals are the major food crops both in terms of the area they are planted and volume of production obtained. They are produced in larger volume compared with other crops because they are the principal staple crops. Cereals are grown in all the regions with varying quantity as shown in the survey results. Out of the total grain crop area, 10,219,443.46 hectares (81.27%) was under cereals where finger millet took up about 456,171.71 hectares (4.46%). In terms of total production, cereals contributed about 253,847,239.63 quintals (87.42%) of the grain production out of which 10, 1705, 92.71 quintals (4%) was contributed by Finger millet (CSA, 2016). It is an important crop

in areas of Tigray, Gojjam, Gonder, and Wollega. It has also become very important crop in the central rift valley of the country including Arsi Negele, Shashemene (Shimelis *et al*., 2009).

There are three dominant and widely occurring varieties (black, red and white) in Ethiopia. The black seeded variety, as described by the farmers, it suited for making local drinks, and has a better fermentation quality, storability and straw quality. The farmers' perception on the ability to tolerate bird attack may be some association with high tannin content of black seeded varieties. On the other hand, the white seeded variety, with no detectable tannin is reported to be highly preferred by birds (Yemane *et al*., 2006). Finger millet has contributed more to agro-morphological attributes such as early maturity, and threshability, as well as end-use attributes such as "Ingira", porridge, grain and milling quality, and farmers associated these traits mainly with the white seeded variety, and to some extent with the red seeded as well (Yemane *et al*., 2006).

Tigray region is located in the northern part of Ethiopia. About 65% of the land in the region is reported to be under cultivation and over 95% of the farmers are small-scale subsistence farmers (BoANRD 1997; Yemane *et al*., 2006). The region is divided into five zones and it is one of the major finger millet growing areas in the country. Finger millet accounts for 11.51% of the crop land allotted to cereals in the Region (CSA, 2016).

2.8. Genetic Markers for Genetic Diversity Analysis

Traditionally, genetic variation is inferred by morphological/ phenotypic variation or the growth response of the organism. Many researchers have also used cytological tools for such studies. Classical methods of establishing genetic diversity and / or relatedness among groups of plants relied upon phenotypic (observable) traits. However, these had two disadvantages: quantitative traits were subject to environmental influences; secondly the levels of polymorphism (allelic variation) that could be looked at were limited. These limitations were significantly overcome by deployment of environment– neutral biochemical makers (Isozymes) and protein electrophoresis and molecular markers that focus directly on the variation controlled by genes or on the genetic material (DNA) (Kephart, 1990; May, 1992). The higher resolution of molecular markers makes them a valuable tool for finger printing and protection of breeder's rights, facilitating appropriate choice of parents for breeding programmemes, analyzing quantitative traits and detection of

Quantitative Trait Loci (QTLs), gene mapping, marker assisted selection and gene transfer, understanding evolutionary pathways and for the assessment of genetic diversity. Hills (1987) recommended that morphological work on large samples combined with molecular analyses on smaller samples maximizes both information and usefulness. Kresovich and McPherson (1992) reported that molecular markers could resolve biological, operational and logistical questions dealing with four broad areas of germplasm characterization: Determination of the correct identity of an individual (whether it i.e. true to type, duplicate etc,), Estimation of the degree of similarity among individuals, Understanding of the hierarchical structure and partitioning of variation among individuals, accessions, populations and species, Identification and detection of the presence of particular alleles in individuals, accessions, populations, chromosomes or cloned DNA segments (Park *et al.,* 2009).

Genotyping of complete or a significant proportion of the gene bank collections provides information to improve the management of plant genetics resources in manifold ways. In this context, storing and managing genotyping data of gene bank materials obtained by using molecular markers is important. The range of molecular markers that can be used on most plant germplasm is quite extensive (Mohan et al., 1997, Gupta and Varshney, 2002). Techniques vary from identifying the polymorphism in the actual DNA sequence to the use of DNA hybridization methods used to identify RFLPs (Restriction Fragment Length Polymorphisms) or the use of PCR based (Polymerase Chain Reaction) technology to find polymorphism using RAPD (Random Amplified Polymorphic DNA), microsatellites such as SSR (Simple Sequence Repeat), ISSR (Inter Simple Sequence Repeat) or combination techniques such as AFLP (Amplified Fragment Length polymorphism). The different methods differ in their cost, ease of application, type of data generated (whether it provides dominant or co-dominant markers) the degree of polymorphism they reveal, the way they resolve genetic difference, and in the taxonomic levels at which they can be most appropriately used (Karp et al., 1997).

2.8.1. Microsatellites Marker

Microsatellites, also referred to as short tandem repeats (STRs), simple sequence repeats (SSRs) or simple sequence length polymorphisms (SSLPs), are tandem repeated motifs of 1-6 bases. They are found in all prokaryotic and eukaryotic genomes and are present in both coding and

non-coding regions (Zane *et al*., 2002). In eukaryotes, these simple sequences are highly repeated and thus offer a nearly unlimited and accessible supply of polymorphisms (Tautz, 1989). They can be analyzed by PCR owing to the fact that the sequences that flank specific SSRs are conserved within a particular species, across species, within a genus and at times across related genera and these conserved areas can be used to design primers for each SSR that is unique (Varshney *et al*., 2002; Parida *et al*., 2009).

Microsatellites are characterized by high degree of length polymorphism (Zane *et al*., 2002). This is attributable to the different number of repeats in the microsatellite regions, which makes it possible to easily and reproducibly detect them by PCR (Kalia *et al*., 2011). These markers are hyper variable, and exhibit wide genomic distribution, co-dominant inheritance, reproducibility, multi-allelic nature and chromosome specific location. These are attributes that make them ideal for plant breeding and genetics (Parida *et al*., 2009). Majority of microsatellites are found in non-coding DNA, either in intergenic regions or in introns, and these comprise the microsatellites that are mostly used as markers (Ellegren, 2004). Their abundance in the genome coupled with the possibility of associating them with many phenotypes makes them powerful for applications in diverse areas in plant genetics (Victoria *et al*., 2011).

2.8.1.1. Classification of Microsatellites

Microsatellites have been classified in a number of ways depending on their size, location in the genome and type of repeat motif. Repeats of one, two, three, four, five and six nucleotides comprise what are classified as microsatellites. Longer repeat units form mini-satellites and in extreme cases, satellite DNA (Ellegren, 2004). The number of nucleotides per repeat unit yields the classification of microsatellites as mono-nucleotides (A)n, di-nucleotide (CA)n, tri-nucleotide (CGT)n, tetra-nucleotide (CAGA)n, penta-nucleotide (AAATT)n or hexa-nucleotide (CTTTAA)n repeats (Kalia *et al*., 2011).

Microsatellites have been further described by Wang and co-workers (2009) as follows; simple perfect repeats, which are tandem arrays of a single repeat sequence such as [AGG]n, simple imperfect arrays, which consist of one or more repeat units of different lengths such as [AAC]n[ACT][AAC]n+1, compound perfect arrays, which are two or more different repeat motifs of the same length such as, [AGG]n [AATC]n and compound imperfect motifs, which are

interrupted by one or more repeats of different lengths such as [GGAT]n[ACT][GTAA]n+1. According to their location in the genome, microsatellites have been classified as nuclear (nuSSR), mitochondrial (mtSSR) or chloroplast (cpSSR).

2.8.2. Inter Simple Sequence Repeats (ISSR)

Inter Simple Sequence Repeat **(**ISSR) are DNA fragments of about 100–3000 bp located between adjacent, oppositely oriented microsatellite regions (Zietkiewicz *et al.,* 1994). Primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16–18 bp). About 10–60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size.

The main advantage of ISSRs is that no sequence data for primer construction are needed; only low quantities of template DNA are required (5–50 ng per reaction), but can have reproducibility problems. The sources of variation in ISSR markers could be mutations at the priming site (SSR), which could prevent amplification of a fragment and thus give a presence/absence polymorphism. Moreover, an insertion/deletion event within the SSR region or the amplified region would result in the absence of a product or length polymorphism depending on the amplifiability of the resulting fragment size. ISSR marker has a wide range of uses, including the characterization of genetic relatedness among populations, genetic fingerprinting, and detection of clonal variation, cultivar identification, phylogenetic analysis and assessment of hybridization (Wang *et al.,* 2009a). Inter Simple Sequence Repeats (ISSRs) have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of indigenous crop species, which includes in *Coffea arabica* (Kassahun Tesfaye *et al.,* 2005) and in lentil (Edossa Fikru *et al.,* 2010).

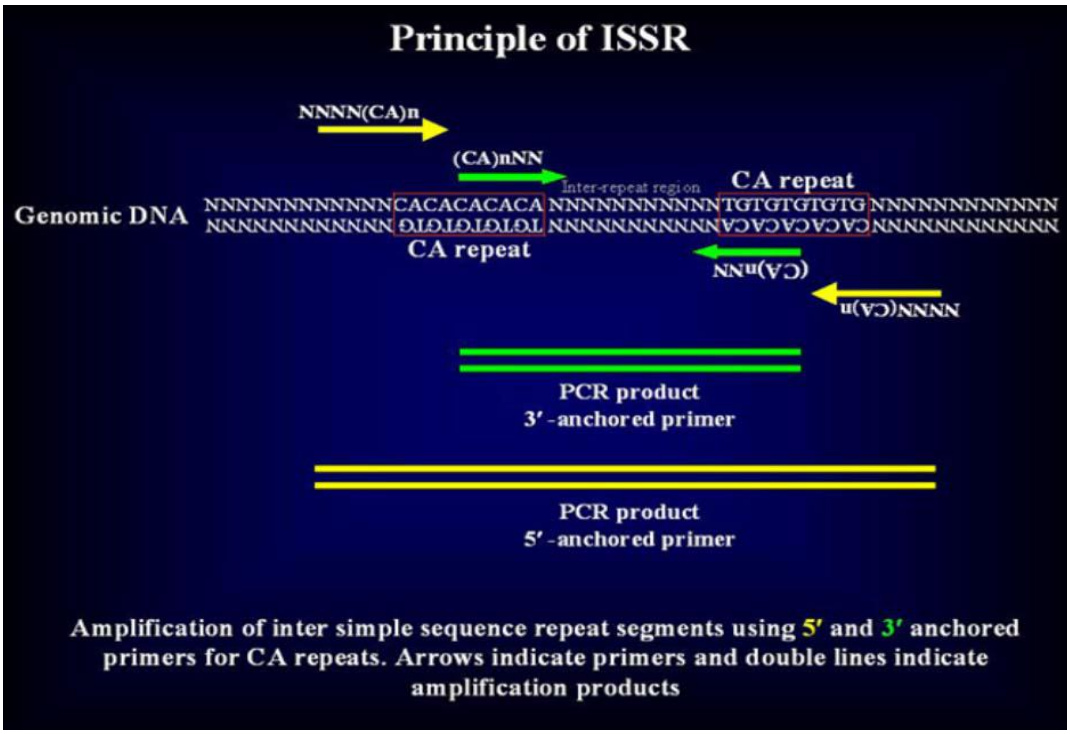

Figure 1: A schematic representation of ISSR-PCR with a single primer (AG)8, unanchored a), 3'-anchored (b) and 5'-anchored (c) targeting a (TC)n repeat used to amplify inter simple sequence repeat region flanked by two inversely oriented (TC)n sequences.

2.8.2.1. Source of Polymorphism of Inter-simple Sequence Repeats (ISSR)

The evolutionary rate of change within microsatellites is considerably higher than most other types of DNA, so the likelihood of polymorphism in these sequences is greater. The source of variability in the ISSRs can be attributed to any one of the following reasons or any combination of these.

I. Template DNA

Slippage of DNA polymerase during DNA replication and failure to repair mismatches is considered as a mechanism for creation and hyper variability of SSRs (Levinson & Gutman, 1987). Mutations at the priming site i.e. SSR could prevent amplification of a fragment, as also in RAPD markers and thus give a presence/absence polymorphism. An insertion/ deletion event within the SSR region or the amplified region would result in the absence of a product or length polymorphism, depending on the amplifiability of the resulting fragment size. Variability in

number of nucleotides within a microsatellite repeat would result in length polymorphisms when using a 5'-anchored primer.

II. ***Nature of primer used***

The extent of polymorphism also varies with the nature (unachored, 3'-anchored, or 5'-anchored) and sequence of the repeats (motif) in the primer employed. When unanchored i.e only the SSRs are used as primers, the primer tends to slip within the repeat units during amplification leading to smears instead of clear bands. Extending the primer (anchoring) with 1 to 4 degenerate nucleotides at the 3' end or 5' end assures annealing only to the ends of a microsatellite in template DNA thus obviating internal priming and smear formation. Secondly, the anchor allows only a subset of the microsatellites to serve as priming sites. When 5' anchored primers are used, the amplified products include the microsatellite sequences and their length variations across a genome and therefore give more number of bands and a higher degree of polymorphism. Usually di-nucleotide repeats, anchored either at 3' or 5' end reveal high polymorphism (Joshi et al., 2000). There is a difference of abundance of SSRs between nuclear and organelle DNA sequences. Taking di- and tri-nucleotides together, one SSR was found every 33Kb in nuclear DNA compared to every 423-Kb of organelle DNA sequence (Wang et al., 1994). In general, primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than primers with other di-, tri- or tetra-nucleotide repeats. (AT) repeats are the most abundant di-nucleotides in plants but the primers based on (AT) would self- anneal and not amplify. Tri and tetra-nucleotides are less frequent and their use in ISSRs is lesser than the di-nucleotides. The (AG) and (GA) based primers have been shown to amplify clear bands in rice (Joshi et al., 2000; Reddy et al., 2000) whereas primers based on (AC) di-nucleotide repeats were found more useful in wheat (Nagaoka & Ogihara, 1997; Kojima et al., 1998) and potato (McGregor et al., 2000).

III. Detection method

The level of polymorphism detected has been shown to vary with the detection method used. Polyacrylamide gel electrophoresis (PAGE) in combination with radioactivity (labelled nucleotide in PCR reaction) was shown to be most sensitive, followed by PAGE with silver staining and then agarose-ethidium bromide system of detection. Markedly higher numbers of

bands were resolved per primer when polyacrylamide was used compared to agarose (Moreno et al., 1998). In a study on trifoliate orange germplasm, silver staining using high quality chemicals could detect all the bands detected by autoradiography (Fang et al., 1997). However, high levels of polymorphism have been detected even when products of ISSR amplification are resolved on agarose gels without radiolabelling (Arcade et al., 2000;& Moore, 2001) Thus, the need for radioactivity can be avoided when many samples have to be screened as in germplasm characterization.

2.9. Origin of Microsatellite Polymorphisms in the Genome

The molecular mechanisms underlying the development of microsatellite variation are still not yet completely understood (Anmarkrud *et al*., 2008). Microsatellite polymorphisms are mainly as a result of variability in length polymorphism rather than in the primary sequence. This is in contrast to unique DNA (Ellegren, 2004). Replication slippage is the primary mechanism reputed to underlie the change in length in microsatellite DNA (Levinson and Gutman, 1987).

Following the commencement of replication of a repeat region, the two strands may dissociate. After realignment occurs, the nascent strand may be out of register, resulting in the nascent strand having a different length from the template strand. An increase in repeat length comes about if a loop is introduced on the nascent strand. On the other hand, if a loop is introduced on the template strand, the consequence is a decrease in repeat length. This is illustrated in Figure 2.

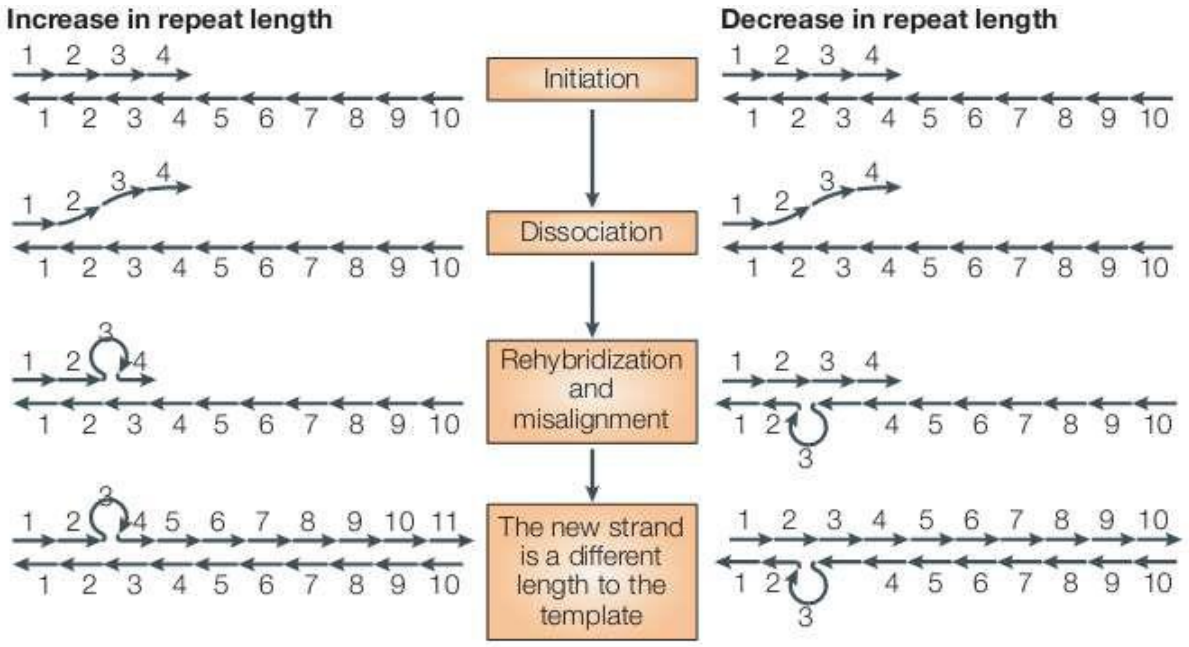

Figure 2: Following the commencement of replication of a repeat region, the two strands might dissociate. Alignment of the nascent strand out of register leads to a different length from the template strand. A loop introduced on the nascent strand results in an increase in repeat length. A loop that is formed in the template strand leads to a decrease in repeat length (Ellegren, 2004).

2.10. Application of Molecular Markers in Finger Millet Research

Various researchers have employed the use of several molecular markers in studying different aspects of finger millet. RAPD markers have been used most frequently in studies on finger millet. This is probably due to their simplicity and applicability (Bardakci, 2000). However, these markers are not reproducible and have problems with data scoring. RFLPs and to a lesser extent, SSRs, have also been used in studying finger millet. Dida *et al*. (2007) employed RFLP, AFLP, EST and SSR markers to generate a skeleton genetic map of finger millet that covered the nine homologous chromosomes of the crop. Parani *et al*., (2001) used PCR-RFLP together with particular restriction enzyme combinations to generate species specific markers for finger millet.

ISSR-PCR is technique that overcomes most of the limitations of RAPD, AFLP, SSR or microsatellite (Zietkiewicz *et al.* 1994; Gupta *et al.* 1994; Wu *et al.* 1994; Meyar *et al.* 1993). In this method SSRs are used as primers to amplify mainly the inter-SSR regions (Reddy *et al.* 2002). ISSR have been successfully used to estimate the extent of genetic diversity at inter and

intra specific level in wide range of crop species which include rice (Joshi *et al.* 2000), wheat (Nagaoka and Ogihara, 1997), finger millet (Salimath *et al.* 1995), Vigna sp. (Ajibade *et al.* 2000) and sweet potato (Huang and Sun, 2000).

Salimath *et al.* (1995) assessed the genome origin and genetic diversity in the genus *Eleusine* by using ISSR to analyse 22 accessions belonging to 5 species of *Eleusine*. Six ISSR primers showed 26 per cent polymorphism in 17 accessions of finger millet from Africa and Asia. This result indicated that very low level of DNA sequence variability in finger millet, but did allow each line to be distinguished. The 16 per cent intraspecific polymorphism exhibited by two analysed accessions of *E. floccifolia* suggested a much higher level of diversity in this species than in *E. coracana. Eleusine floccifolia* and *E. compressa* were found to be the most divergent among the species examined.

Fakrudin *et al*. (2007) conducted diversity studies using RAPDs in twelve accessions from different geographical regions and reported 85.82% polymorphism across the whole set of samples. They observed fewer polymorphisms for Indian accessions compared to the more diverse African accessions. Babu *et al*. (2007) used 32 finger millet genotypes to assess diversity using RAPDs and established that RAPDs were useful in discriminating between finger millet genotypes, reporting polymorphism of between 44.5 and 100%. Panwar *et al*. (2010) did a comparative evaluation of genetic diversity using RAPDs, SSRs and cytochrome P450. The level of polymorphism they reported for each of the markers was 49.43, 50.2 and 58.7% for RAPDs, SSRs and cytochrome P450, respectively in the 52 genotypes they studied.

Sinha and Pande (2010) did a finger printing study of finger millet using microsatellites. They hailed the success of microsatellites in uncovering variation in finger millet, which is a self-pollinated plant and thus shows little or no polymorphism with other markers. Dida *et al*. (2008) used 45 SSR markers to evaluate genotypic variation among 79 finger millet accessions across their range of cultivation in Africa and Asia. This so far has been the largest study conducted in terms of number of markers and number of accessions studied. The study predicted origins of breeding material unknown prior to the study. The study also substantiated the theory that finger millet was domesticated in Africa and then introduced to India. It is worth noting that all the finger millet SSRs used for the various studies were obtained from among the 82 SSRs published by Dida *et al*. (2007).

3. Objectives of the Study

3.1. General Objective

The general objective of this study was to determine the genetic variability and patterns of diversity of finger millet (*Eleusine coracana*) with varying seed coat color of two zones in Tigray Regional State.

3.2. Specific Objectives

Specifically, this study aimed to:

- study the genetic diversity of finger millet (*Eleusine coracana*) with varying seed coat color, namely red, white and black from two zones in Tigray Regional State;
- determine the relationship within and among finger millet accessions with various seed coat color from two zones of Tigray;
- identify finger millet populations with higher variability for future conservation and improvement.

4. MATERIALS AND METHODS

The present investigation was conducted at Post-graduate School greenhouse of Addis Ababa University college of Natural and computational Science in May 2017, and the molecular studies were conducted at Plant Genetics Laboratory of the Microbial, Cellular, and Molecular Biology Department, Addis Ababa University. The details of the study area, the materials and the methods used to carry out the experiment and statistical procedures followed are described in this chapter.

4.1. Description of Study Area

Tigray region is located in the northern part of Ethiopia. About 65% of the land in the region is reported to be under cultivation and over 95% of the farmers are small-scale subsistence farmers (BoANRD, 1997; Yemane *et al*., 2006). The region is divided into five zones and it is one of the major finger millet growing areas in the country. Finger millet accounts 11.51% of the crop land allotted to cereals in the Region, and 68.91%, 13.09%, 13.82%, 3.53%, and 0.64% of the crop land allotted to cereals in North-west, Central, Western, Eastern and Southern zones of Tigray, respectively (CSA, 2016). This study focused on four woredas of two zones of Tigray, from Central Zone Wereda Ahiforom and Wereda Mereb-Leke and from North-west Zone Wereda Asgede-Tsimbla and Wereda Laela-Adyabo where the Finger millet is grown (Figure 3).

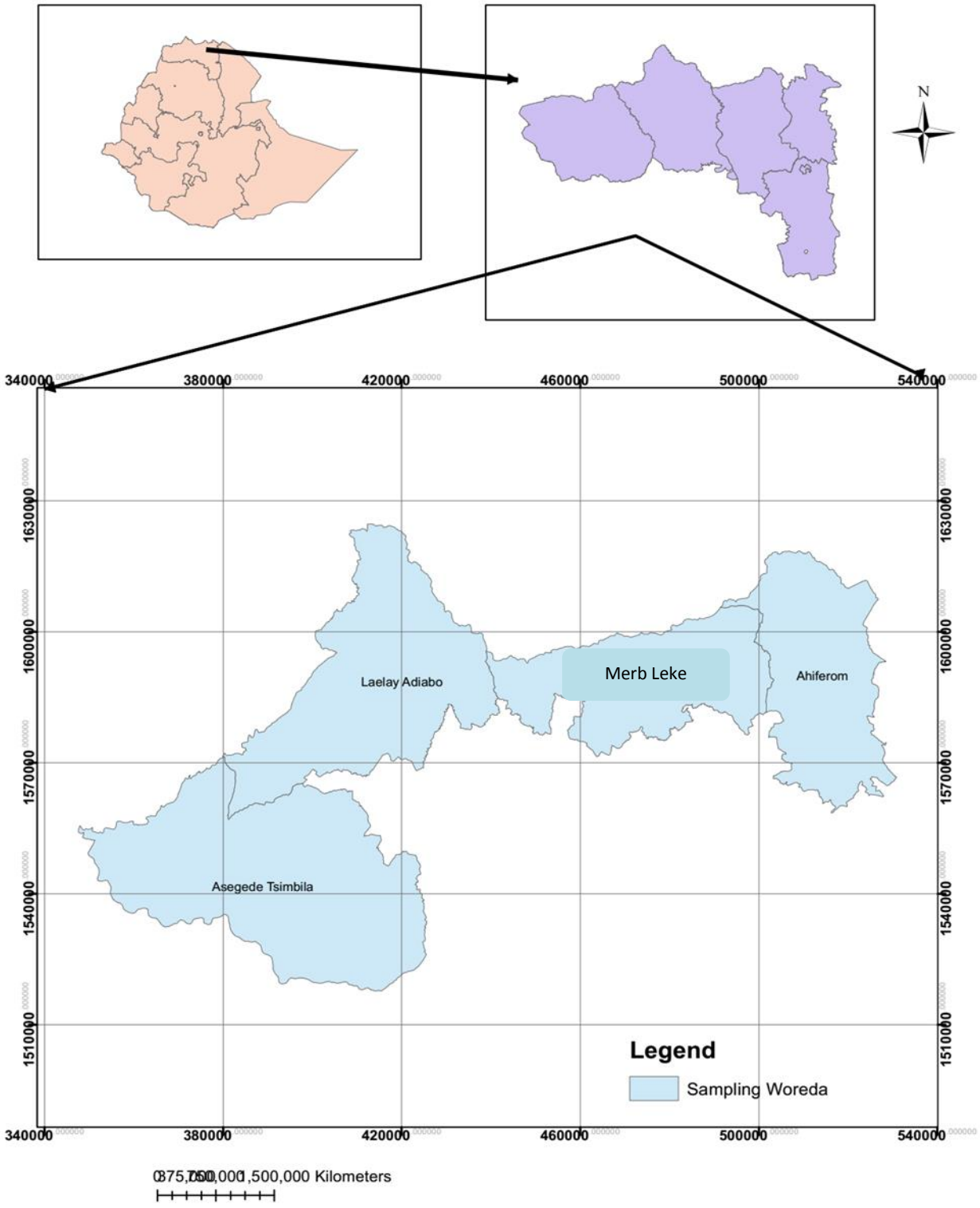

Figure 3: Map of the study area (Source:Ethiopian Maping Agency)

4.2. Plant Materials and Germination Conditions

For this study, a total of 72 finger millet accessions were collected from two Woredas of Central Zone of Tigray and two woredas of North West Zone of Tigray. Specifically, 18 each Finger millet accessions with variable seed coat color were collected from Woreda Ahiforom, and Wereda Mereb-Leke (both found in Central Zone of Tigray); and 18 each Finger millet accessions from Woreda Asgede-Tsimbla and Laelay Adiabo (both found in North West Zone of Tigray). From each woreda, 6 red seed coat, 6 black seed coat, and 6 white seed coat accessions of finger millets were collected directly from the farmer's field. Seeds of these varieties were sown in plastic pots filled with approximately 5 kg normal soil and the seedlings were grown for about 20 days in the green house of Addis Ababa University; they were planted and germinated separately in the plastic pots (Table 2).

Table 2: List and number of accessions of Finger millet (*E. coracana*) obtained from Tigray for the study

SN	Acc.name	Region	Wereda	S.C	Altitude	SN	Acc.name	Region	Wereda	S.C	Altitude
1	BA-1	Tigray	Ahiforom	Black	2118	37	BAT-37	Tigray	Asgede-Tsimbila	Black	1089
2	BA-2	Tigray	Ahiforom	Black	2064	38	BAT-38	Tigray	Asgede-Tsimbila	Black	1108
3	BA-3	Tigray	Ahiforom	Black	2020	39	BAT-39	Tigray	Asgede-Tsimbila	Black	1515
4	BA-4	Tigray	Ahiforom	Black	1930	40	BAT-40	Tigray	Asgede-Tsimbila	Black	1133
5	BA-5	Tigray	Ahiforom	Black	1890	41	BAT-41	Tigray	Asgede-Tsimbila	Black	1131
6	BA-6	Tigray	Ahiforom	Black	1888	42	BAT-42	Tigray	Asgede-Tsimbila	Black	1702
7	RA-7	Tigray	Ahiforom	Red	2118	43	RAT-43	Tigray	Asgede-Tsimbila	Red	1089
8	RA-8	Tigray	Ahiforom	Red	2064	44	RAT-44	Tigray	Asgede-Tsimbila	Red	1108
9	RA-9	Tigray	Ahiforom	Red	2020	45	RAT-45	Tigray	Asgede-Tsimbila	Red	1515
10	RA-10	Tigray	Ahiforom	Red	1930	46	RAT-46	Tigray	Asgede-Tsimbila	Red	1133
11	RA-11	Tigray	Ahiforom	Red	1890	47	RAT-47	Tigray	Asgede-Tsimbila	Red	1131
12	RA-12	Tigray	Ahiforom	Red	1888	48	RAT-48	Tigray	Asgede-Tsimbila	Red	1702
13	WA-13	Tigray	Ahiforom	White	2118	49	WAT-49	Tigray	Asgede-Tsimbila	White	1089
14	WA -14	Tigray	Ahiforom	White	2064	50	WAT-50	Tigray	Asgede-Tsimbila	White	1108
15	WA-15	Tigray	Ahiforom	White	2020	51	WAT-51	Tigray	Asgede-Tsimbila	White	1515
16	WA-16	Tigray	Ahiforom	White	1930	52	WAT-52	Tigray	Asgede-Tsimbila	White	1133

17	WA -17	Tigray	Ahiforom	White	1890	53	WAT-53	Tigray	Asgede-Tsimbila	White	1131
18	WA 18	Tigray	Ahiforom	White	1888	54	WAT-54	Tigray	Asgede-Tsimbila	White	1702
19	BM-19	Tigray	Mereb-Leke	Black	1386	55	BLA-55	Tigray	Laelay-Adyabo	Black	1735
20	BM-20	Tigray	Mereb-Leke	Black	1380	56	BLA-56	Tigray	Laelay-Adyabo	Black	1821
21	BM-21	Tigray	Mereb-Leke	Black	1346	57	BLA-57	Tigray	Laelay-Adyabo	Black	1811
22	BM-22	Tigray	Mereb-Leke	Black	1373	58	BLA-58	Tigray	Laelay-Adyabo	Black	1816
23	BM-23	Tigray	Mereb-Leke	Black	1492	59	BLA-59	Tigray	Laelay-Adyabo	Black	1723
24	BM-24	Tigray	Mereb-Leke	Black	1367	60	BLA-60	Tigray	Laelay-Adyabo	Black	1719
25	RM-25	Tigray	Mereb-Leke	Red	1386	61	RLA-61	Tigray	Laelay-Adyabo	Red	1735
26	RM-26	Tigray	Mereb-Leke	Red	1380	62	RLA-62	Tigray	Laelay-Adyabo	Red	1821
27	RM-27	Tigray	Mereb-Leke	Red	1346	63	RLA-63	Tigray	Laelay-Adyabo	Red	1811
28	RM-28	Tigray	Mereb-Leke	Red	1373	64	RLA-64	Tigray	Laelay-Adyabo	Red	1816
29	RM-29	Tigray	Mereb-Leke	Red	1492	65	RLA-65	Tigray	Laelay-Adyabo	Red	1723
30	RM-30	Tigray	Mereb-Leke	Red	1367	66	RLA-66	Tigray	Laelay-Adyabo	Red	1719
31	WM-31	Tigray	Mereb-Leke	White	1386	67	WLA-67	Tigray	Laelay-Adyabo	White	1735
32	WM-32	Tigray	Mereb-Leke	White	1380	68	WLA-68	Tigray	Laelay-Adyabo	White	1821
33	WM-33	Tigray	Mereb-Leke	White	1346	69	WLA-69	Tigray	Laelay-Adyabo	White	1811
34	WM-34	Tigray	Mereb-Leke	White	1373	70	WLA-70	Tigray	Laelay-Adyabo	White	1816
35	WM-35	Tigray	Mereb-Leke	White	1492	71	WLA-71	Tigray	Laelay-Adyabo	White	1723
36	WM-36	Tigray	Mereb-Leke	White	1367	72	WLA-72	Tigray	Laelay-Adyabo	White	1719

**N^{o} Acc. = Number of accessions, SC= Seed coat color, B= Black seed coat color, R= Red seed coat color, W= White seed coat color, A= Ahiforom, M= Mereb-leke, AT= Asgede-tsimbla, LA= Laelay-adyabo BA; Black Ahiforom (1-6), RA; Red Ahiforom (7-12), WA; White Ahiforom (13-18), BM; Black Mereb-Leke (19-24), RM; Red Mereb-Leke (25-30), WM; White Mereb-Leke (31-36), BAT; Black Asgede-tsimbla (37-42), RAT; Red Asgede-tsimbla (43-48), WAT; White Asgede-tsimbla (49-54), BLA; Black Laelay-adyabo (55-60), RLA; Red Laelay-adyabo (61-66), WLA; White Laelay-adyabo (67-72).*

4.3. Isolation of Genomic DNA from Young Leaves of Finger Millet

20 day old young leaves (~1g) of finger millet were dried in silica gel and equal amounts (0.4g) of the dried leaves placed in 2 ml eppendorf tubes and immediately these tubes were then processed in a Mixer Mill grinding machine (Retsch MM400, Germany) to obtain fine powder. Total DNA was extracted from three representative plant of each accessions of the young leaves of seventy two sample finger millet different seed coat color by Cetyl Tri methyl Ammonium Bromide (CTAB) method (2 % Cetyltrimethyl ammonium Bromide, 1 % polyvimylpyrolidone, 100 mM Tris: PH=8, 20 mM EDTA, 1.4 M NaCl) and 0.03 M beta-Mercapto-ethanol extraction protocol, previously as described by Borsch *et al.,* (2003), with some modifications appendix (1), followed by quantification of DNA using a spectrophotometer. The experiment was conducted at Plant Genetics Laboratory of the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University.

4.4. DNA Quality and Amount Testing

Quantification of purified DNA was performed using UV visible spectrophotometer (NanoDrop™2000/2000c) at 260 and 280 nm wave lengths, and the ratio of absorbance 260/280 was calculated. 5µl of all DNA extracts were electrophoresed (Bio Rad sub cell, USA). After electrophoresis, the band intensity of genomic DNA was visualized on gel documentation unit (Bio-Rad, USA). These gels also provided a visual measure of purity and integrity of DNA. In the end, the samples were diluted to a final concentration of 50ng/µl. Agarose (0.86g) was added to 50 ml of 1X TBE buffer and was melted by heating the solution in microwave oven. The solution was cooled to about 55-60 °C, and 2µl of ethidium bromide (10 mg/ ml) was added to it. The gel solution was poured into the gel casting unit after keeping the comb in the assigned slots, and the gel was allowed to solidify at room temperature. Gel was placed in the electrophoresis apparatus in such a way that the end with wells is in line with the cathode. The apparatus was filled with 1X TBE buffer in order to submerge the gel in the buffer to prevent the entry of air bubbles while removing the gel combs. The 25µl PCR products to be analyzed were mixed with 2.5µl loading (tracking) dye (6X) and loaded carefully in the wells of the gel. The unit was connected to a power pack, and electrophoresis was carried out at 110 volts for one hour. The power supply was switched off when the front dye was about 5cm away from positive end

(anode). The amplified PCR products were observed under UV trans-illuminator in gel documentation system (Bio-Rad, USA), and image was captured (Figure 4).

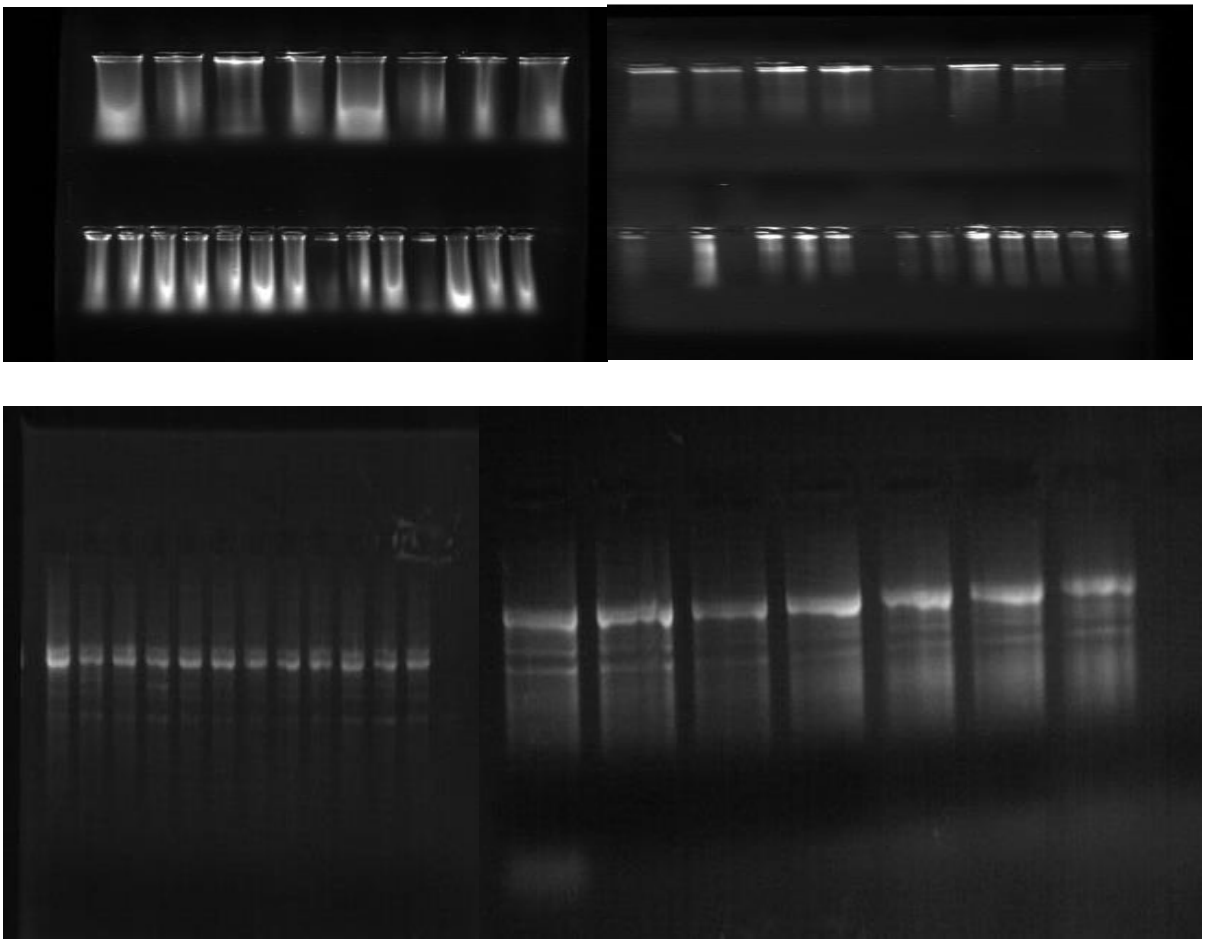

Figure 4: Genomic DNA test gel result

4.5. ISSR PCR

A set of 10 ISSR primers, i.e. commercial kits obtained from Primer kit 900 (UBC 900), were used. Out of 10 ISSR primers, 6 primers were scorable. Three individuals were selected from each population with 1:5 dilutions to screen the primers. A total of six polymorphic and reproducible ISSR primers were selected after testing and screenings. The details of the ISSR primers sequences and melting temperature (Tm) were presented in Table 3, for their annealing temperature with respective sequences and their amplification pattern.

Table 3: Sequences details of ISSR primers used for diversity study in finger millet

No	Primer	Primer Sequence(5'-3')	Annealing Temperature (Ta)	Motif
1	ISSR-810	5'GAGAGAGAGAGAGAGAT-3'	45 ºC	Di-nucleotide
2	ISSR-811	5'-GAGAGAGAGAGAGAGAC-3'	48 ºC	Di-nucleotide
3	ISSR-834	5'-AGAGAGAGAGAGAGAGYT-3'	47 ºC	Di-nucleotide
4	ISSR-857	5-ACACACACACACACACYG-3'	49 ºC	Di-nucleotide
5	ISSR-866	5'-CTCCTCCTCCTCCTCCTC-3'	55 ºC	Tri-nucleotide
6	ISSR-880	5'-GG AGA GG AG AGG AGA-3'	45 ºC	Penta-nucleotide

Source: Primer kit 900 (UBC 900); R = Purines (A or G), Ta= annealing temperature, Y = (C,T);

Amplification was performed in a 0.5 ml PCR tube having 25µl PCR reaction mixture volume as described by Gupta *et al*. (2010), with some modifications. The composition of PCR mixture is presented in (Table 4). Polymerase chain reaction (PCR) amplifications were performed in Biometra 2003 T3 Thermo cycler programmed to run the following temperature profile: a preheating and initial denaturation for 4 minutes at 94ºC, then 15 seconds denaturation at 94ºC, 1 minute primer annealing at 45 / 48/55ºC (based on primers used), 1.30 minutes extension at 72 ºC for 40 cycles and 7 minutes' final extension at 72 ºC with holding temperature at 4 ºC. Each PCR reaction of ISSR markers had a final reaction volume of 25µl, containing 4 mM dNTPs, 10x PCR buffer (100 mM Tris HCl (PH8.8), 500 mM KCl, 0.1% Tween 20 and 15 mM MgCl2), 3 mM MgCl2, 8 pmol primer, 2 U Taq polymerase and 10-100 ng template DNA. A negative control, in which the template DNA was replaced by double distilled water, was also included in each round of reactions to check for absence/presence of contamination. The PCR products were stored at 4ºC until loading on the gel for electrophoresis. Amplification products were separated by electrophoresis in 1.67 % (w/v) agarose gels.

Table 4: Composition of PCR mixture for ISSR analysis

No.	Components	Stock concentration	No. Volume of PCR reaction mixture per tube (25µl)
1	*Taq* DNA polymerase Buffer(BD)	10X	3µl
2	$MgCl_2$	25mM	4µl
3	dNTPs mix	20Mm of each	1µl
4	Primer	1 M	0.4µl
5	*Taq* DNA polymerase	2U/ µl	0.4µl
6	Template DNA	10ng /µl	2µl (20ng)
7	Sterile distilled water	-	15.2µl
8	**Total volume**	-	**25µl**

4.5. Data Analysis

The clearly resolved amplicons of finger millet genotypes were scored manually for their presence (1) and absence (0) or ambiguous (?), and each band was regarded as a locus in the data sheet. Data were analyzed and similarity matrix was constructed from binary data with Dice similarity coefficients, which were calculated as per model suggested by Nei and Li (1979). Unweighted Pair Group Method using Arithmetic Averages (UPGMA) was employed for cluster analysis. The analysis was carried out using the software package NTSYSpc 2.02i (Rohlf, 1998). Based on generated binary data (0 and 1) matrix, different softwares were used for the analysis. POPGENE version1.32 software (Yehe *et al.,* 1999) was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, gene diversity (h) and Shannon–Weaver diversity index (I). Analysis of molecular variance (AMOVA) was used to calculate variation among and within population with the use of Areliquin version 3.01 (Excoffier *et al.,* 2006).

5. RESULTS

5.1. ISSR Polymorphism

The 72 finger millet genotypes with varying seed coat colors that are black, red, and white were assessed for amplifications pattern using 10 ISSR primers. Among these, 6 primers produced polymorphic amplicons and were selected for genetic diversity analysis of the crop. The six ISSR primers produced a total of 45 reproducible fragments with an average of 7.5 fragments per primer. Among these, 30 were polymorphic for more than one variety and 15 amplicons were monomorphic (Table 5).

Table 5: Details of the ISSR primers used and polymorphism status

No	Details	Number
1	Total number of primers used	10
2	Number of polymorphic primers	6
3	Total number of loci	45
4	Total number of polymorphic loci	30
5	Total number of monomorphic loci	15
6	Percentage of polymorphic loci	66.67

The polymorphism of ISSR primer fragments shown in table 5, indicate that among 10 primers, 6 were highly polymorphic while 4 primers showed low polymorphism. The range of amplified fragments generated by each primer varied from 4 to 7. Primer 834, and primer 810 produced 4 fragments, and primer 880 produced maximum of 7 amplified fragments whereas the primers 811, 857, and 866, produced 5 fragments each. The fragment size amplified with these primers was in the range of 300 to 2000 base pairs. The amplification pattern of some ISSR primers is shown in (Figure 5).

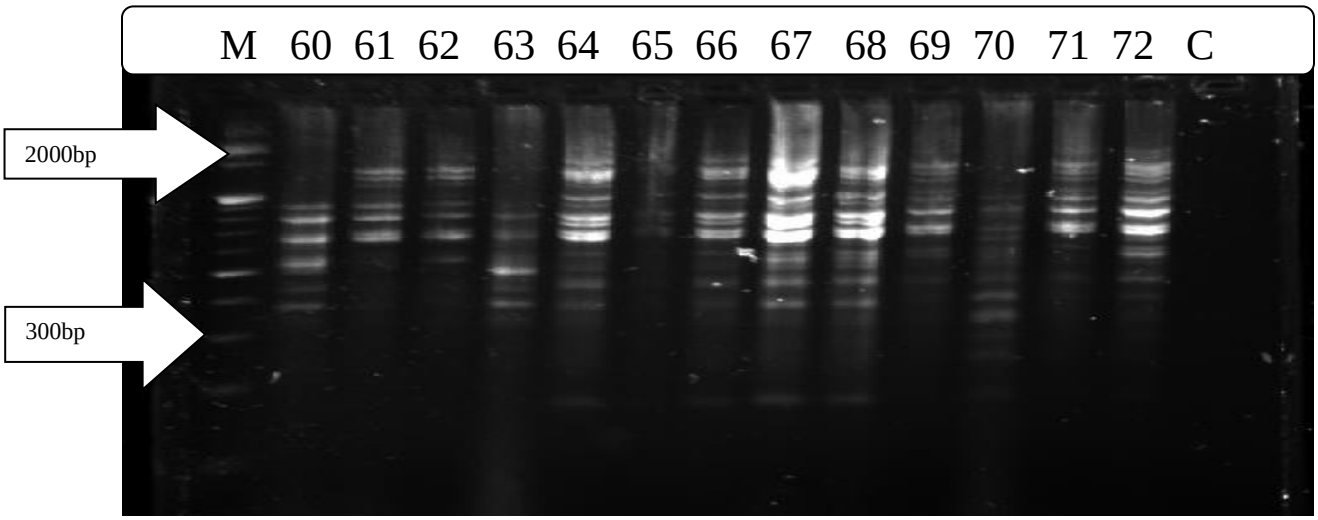

Figure 5: Banding pattern of primer 880 in finger millet accessions. M represents a 100 bp DNA ladder as a standard molecular marker; 60-72 represents accession code, C represents control (a PCR reaction mix without template DNA).

Out of the 45 Loci 30 fragments were polymorphic which accounts for 66.67 %, and 15 loci were monomorphic (Table 6). Among the polymorphic loci, maximum percentage of polymorphic loci (87.5 %) was generated by ISSR-880 primer followed by ISSR-811 and ISSR-866 which accounted 71.43% each. Primer 857 generated 62.5% while ISSR-810 and ISSR-834 generated the least percentage of polymorphic loci of 57.14%, and 50%, respectively. Furthermore, penta-nucleotide primer (ISSR-880) also generated high number of percent polymorphism within population as compared to the di- nucleotide, and tri-nucleotide, showing that the penta-nucleotide ISSR-880 revealed more polymorphism.

Table 6: Number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), gene diversity (h) and Shannon information index (I) calculated for each primer.

No	Primer	NPL	PPL	H ± SD	I±SD
1	ISSR-810	4	57.14%	0.23± 0.22	0.34± 0.32
2	ISSR-811	5	71.43%	0.29± 0.19	0.42± 0.29
3	ISSR-834	4	50%	0.19± 0.2	0.28± 0.3
4	ISSR-857	5	62.5%	0.26± 0.21	0.38± 0.31
5	ISSR-866	5	71.43%	0.29± 0.19	0.42± 0.29
6	ISSR-880	7	87.5%	0.35± 0.14	0.51± 0.21

***NPL** = Number of polymorphic Loci; **PPL** = Percent of polymorphic Loci; **h** = gene diversity; **I** = Shannon's information index and **SD**= standard deviation*

The gene diversity (h) indexes of the four populations with varying seed coat colors for all primers used were calculated and the values ranged from 0.26 for Ahiferom, Mereb-Leke, and Asgede-tsimbla to 0.27 for Laelay-adyabo according to their geographical locations. Besides, when the finger millet populations were grouped in to seed coat color (black, red, and white), the gene diversity (h) was 0.27 for black seed coat color and 0.26 each for red and white colors. In addition, the same diversity patterns were also observed for Shannon's information index whereby the least was obtained from Ahiferom, Mereb-Leke, and Asgede-tsimbla (0.38) while highest diversity was displayed by populations from Laelay-adyabo (0.39). Among finger millet groups (delineated on seed color) the Shannon index had shown the same variability (0.39) (Table 7).

Table 7: The gene diversity (h), and Shannon's information (I) Index, using data generated from all the six primers. The analysis was carried out at population level and grouping made based on Seed coat color of the crop

No	Population	H ± SD	I±SD
1	Ahiforom	0.26±0.20	0.38±0.29
2	Mereb-Leke	0.26±0.20	0.38±0.28
3	Asgede-tsimbla	0.26±0.20	0.38±0.28
4	Laelay-adyabo	0.27±0.20	0.39±0.29
Groups			
1	Black seed coat color	0.27±0.19	0.39±0.29
2	Red seed coat color	0.26±0.19	0.39±0.28
3	White seed coat color	0.26±0.20	0.39±0.28

***h** = gene diversity; **I** = Shannon's information index and **SD**= standard deviation*

5.2. Cluster Analysis

Dendrogram constructed with NTSYSpc 2.02i on the basis of ISSR polymorphism revealed the pattern of relatedness among 72 finger millet genotypes. Clustering analysis based on Un-weighted Pair Group Method using Arithmetic Averages (UPGMA) using NJ similarity coefficient grouped the 72 finger millet genotypes into three mega-clusters. Cluster II had highest number of genotypes (47 genotypes) followed by cluster III (13 genotypes), and cluster I (12 genotypes). The major clusters I, II, and III were further grouped into sub-clusters. The details of the genotypes included in each sub-cluster are presented in Table 8 and depicted in dendrogram (Figure.6).

The results presented in table 8 show that, cluster II was the largest consisting of 47 genotypes sub grouped into 4 sub cluster while cluster I, and III were again grouped into 2 sub-clusters. Similarity, analysis of the ISSR markers revealed moderate to high diversity among the finger millet genotypes, with the similarity index value ranging from 0.64 to 1 (Figure 6.). Maximum diversity was observed between accessions BA-3 and BA-6 (1.), followed by BAT-41 and BAT-42 (1), and RAT-44 and RAT-45 (1). Most of the genotypic combinations showed similarity

index above 0.82, whereas the 72 genotypic combinations showed similarity index between 0.64 to 1 indicating high similarities.

Table 8: Distribution of finger millet genotypes into different clusters based on analysis

No of Cluster	Sub-cluster	No of SC per cluster	No of accessions	Accessions	No of accessions per population
I	IA	B=6/8 R=2/8	8	BA-1, BA-4, RA-8, BA-2, BA-3, BA-6, RA-7, BA-5	A=8/8
	IB	R=4/4	4	RA-9, RA-11, RA-10, RA-12	A=4/4
II	IIA	B=6/11 W=5/11	11	WA-14, WA-17, BM-19, WA-15, WA-16, BM-20, BM-21, WA-18, BM-24, BM-22, BM-23	A=5/11 M=6/11
	IIB	B=6/12 R=6/12	12	BAT-37, BAT-38, BAT-39, BAT-40, BAT-41, RAT-44, BAT-42, RAT-45, RAT-46, RAT-47, RAT-43, RAT-48	AT=12/12
	IIC	R=6/12 W=6/12	12	RLA-61, RLA- 62, RLA-64, RLA-66, WLA-67, WLA-72, WLA-71, WLA-70, WLA-68, RLA-65, WLA-69, RLA-63	LA=12/12
	IID	B=6/12 W=6/12	12	WAT-49, WAT-51, WAT-52, WAT-54, WAT-53, BLA-57, BLA-59, WAT-50, BLA-56, BLA-58, BLA-60, BLA-55	AT=6/12 LA=6/12
III	IIIA	R=5/12 W=7/12	12	WA-13,WM-31, WM-32, WM-36, RM-26, WM-34, RM-27, RM-28, RM-29, WM-35, RM-30, WM-33	A=1/12 M=11/12
	IIIB	R=1/1	1	RM-25	M=1/1
Total			**72**		

***BA**= Black Ahiforom (1-6), **RA**= Red Ahiforom (7-12), **WA**= White Ahiforom (13-18), **BM**= Black Mereb-Leke (19-24), **RM**= Red Mereb-Leke (25-30), **WM**= White Mereb-Leke (31-36), **BAT**= Black Asgede-tsimbla (37-42), **RAT**= Red Asgede-tsimbla (43-48), **WAT**=White Asgede-tsimbla (49-54), **BLA**=Black Laelay-adyabo (55-60), **RLA**=Red Laelay-adyabo (61-66), **WLA**= White Laelay-adyabo (67-72), **A**= Ahiform, **AT**= Asgede-tsimbla, **LA**= Laelay-adyabo, **M**=Mereb-leke **SC**= Seed coat color*

The individual based UPGMA clustering of an overall analysis showed strong clustering of individuals with respect to their population and seed coat color except few intermixed populations. The individual based dendrogram constructed using UPGMA clearly identified three major clusters (I, II, and III) using Jaccard's similarity coefficient of around 99 % (Figure 6). The first cluster was composed of 12 accessions collected from Ahiforom wereda with black and red seed coat color. The second major cluster consisted of 47 accessions were white seed coat color which were collected from wereda Ahiforom, black seed coat color from wereda Mereb-leke, black, red, and white seed coat colors from wereda Asgede-tsimbla, and wereda laelay-adyabo. The third major cluster consisted of 13 accessions collected from wereda Mereb-leke with red and black seed coat color (Figure 6). Most individuals from all populations with varying seed coat color formed their own cluster, while only few of the individuals were distributed all over the tree.

The dendrogram derived from NJ analysis of the whole ISSR data showed three distinct clusters (major cluster-I, cluster-II, and cluster-III). Major cluster I formed two sub-clusters (I-A and I-B) and Major cluster II formed four sub-clusters (II-A, II-B, II-C, and II-D), and major cluster-III formed two sub-clusters (III-A, and III-B) as shown in Figure 6. Major cluster I, and III were dominated by accessions collected from woreda Ahiforom, and woreda Mereb-leke respectively, while major cluster II consisted of mixed accessions with varying seed coat colors.

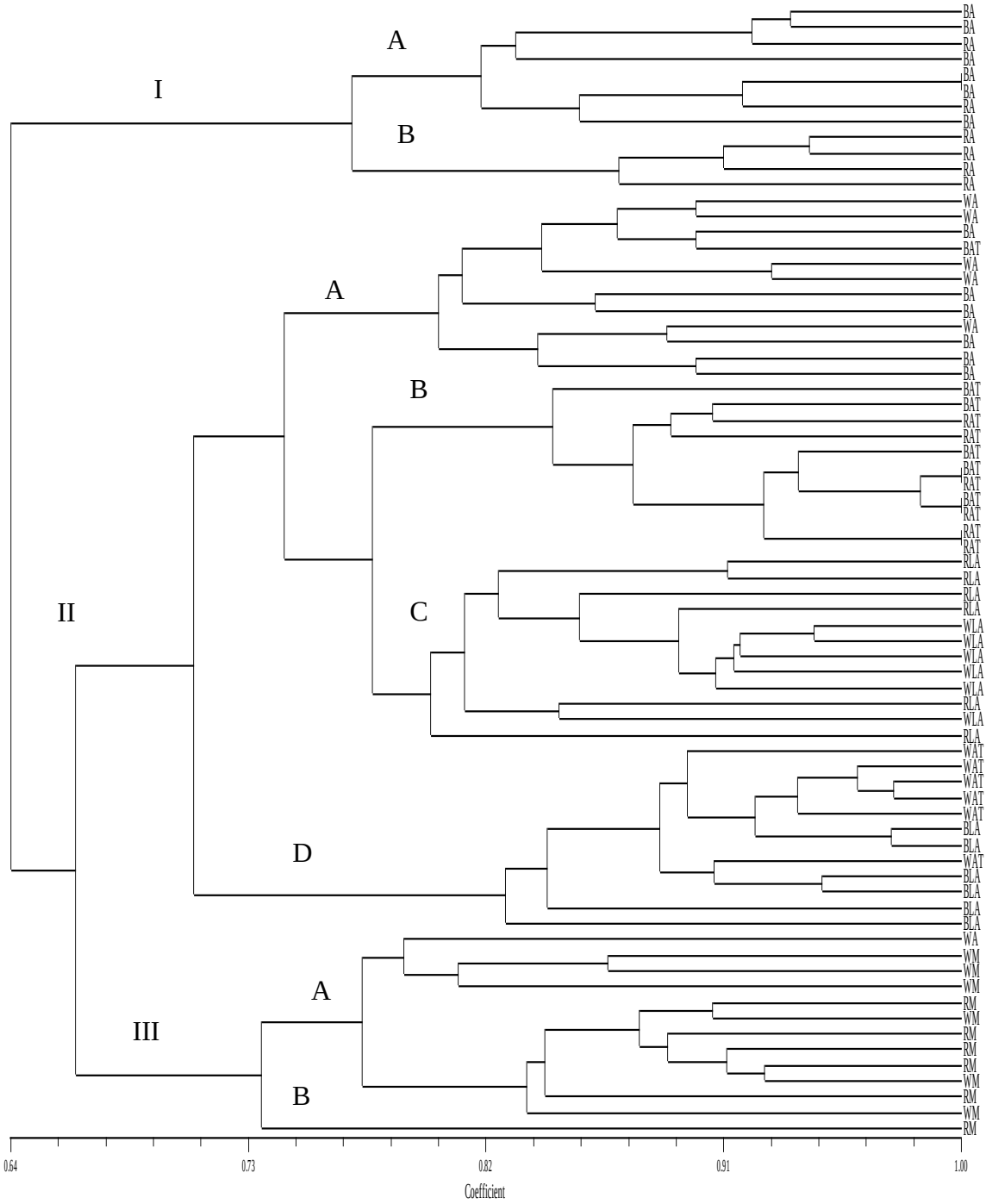

Figure 6: **UPGMA based dendrogram of 72 finger millet genotypes using ISSR markers**

The dendrogram derived from NJ analysis of the whole ISSR data showed two distinct clusters (major cluster-I and major cluster-II). Major cluster I formed two sub-clusters (sub-clusters I-A and I-B) and Major cluster II had not sub-clusters (Figure 7). Major cluster I was dominated by

accessions collected from Ahiforom, Asgede-tsimbla, and Laelay-adyabo, while major cluster II consisted of accessions from Mereb-leke.

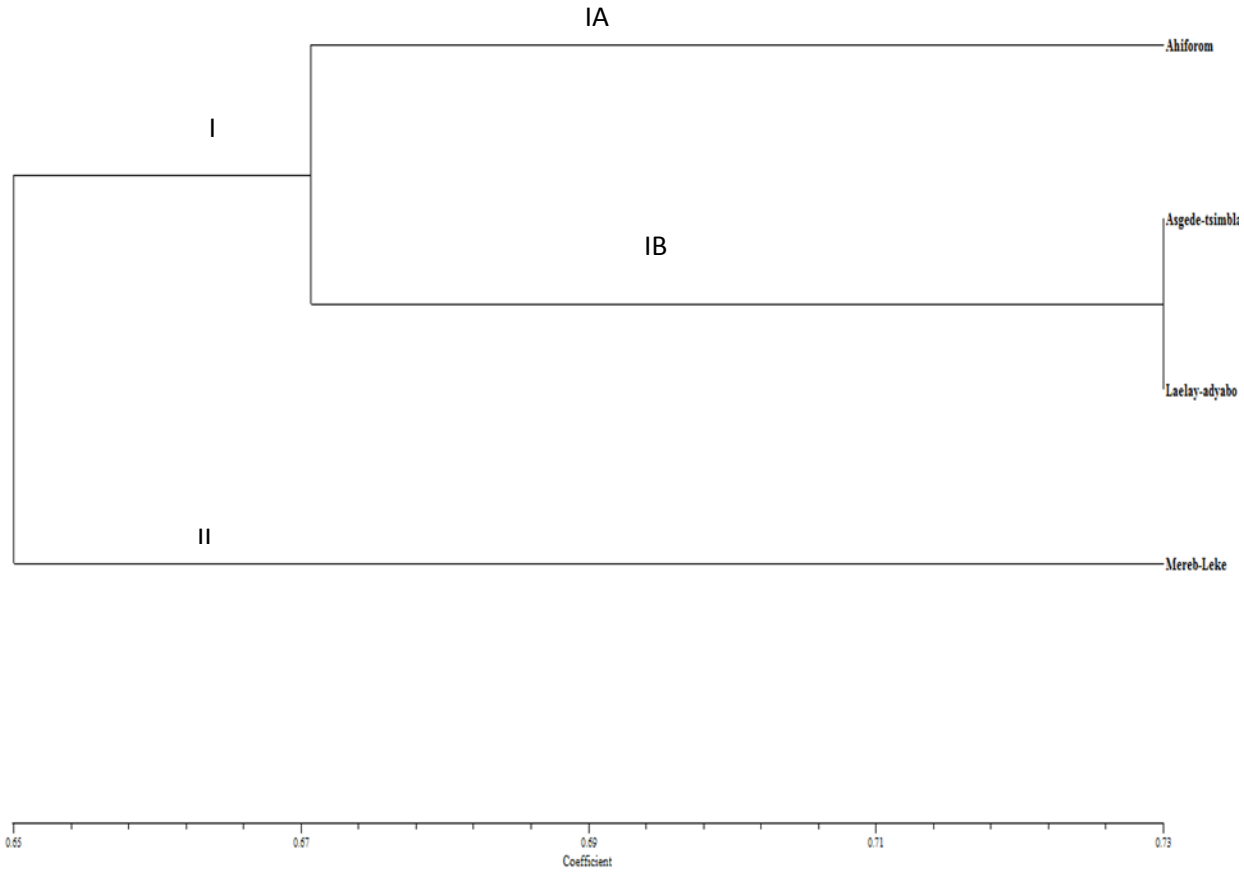

Figure 7: UPGMA based dendrogram of 4 populations of finger millet using ISSR markers

5.3. Principal Coordinates Analysis (PCoA)

Principal coordinate analysis (PCoA) was carried out on all the ISSR data obtained from 72 accessions with varying seed coat color. The analysis was carried out using GeneAlex software's by employing Jaccard's coefficients of similarity. The first three coordinates of the PCoA with values of 9.54, 8.43, and 7.49, and variation percentages of 9.54%, 17.97%, and 25.46%, respectively, were used to show the grouping of all of the accessions using coordinates (Figure 8). The geographical based and color based group clustering observed in two dimensional principal coordinate analysis indicated the intermixing of finger millet accessions. The result agrees with clustering pattern observed in UPGMA. In the plot most of the individual accessions

that represent four different populations spread all over the plot form clusters. Overall, not clear grouping was observed among accessions (Figure 8). The same result was observed in grouping based on their color (Figure 9).

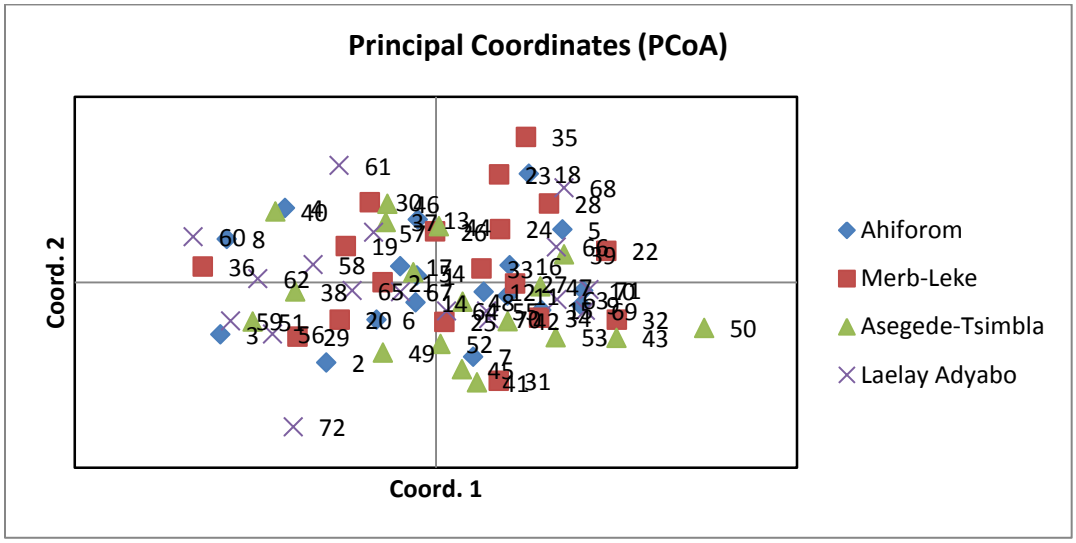

Figure 8: Principal Coordinate Analysis (PCoA) finger millet accession based on geography

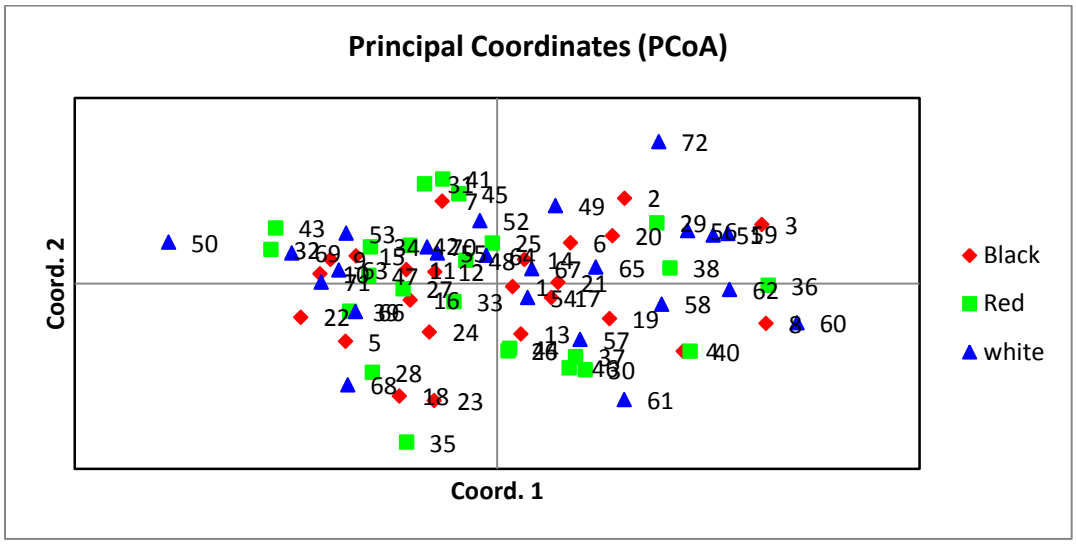

Figure 9: Principal Coordinate Analysis (PCoA) data based on color differentiations

5.4. Analysis of Molecular Variance (AMOVA)

AMOVA was carried out using all 45-ISSR bands generated by Finger millet (*E. coracana)* population with and without grouping (Table 9). AMOVA without grouping populations revealed that maximum percentage of variations (98%) is attributed to the within population while 2% is due to the among population variations. There were a significant genetic differences between the three groups as well as between the four populations of Finger millet *(E. coracana)* (AMOVA; $p<0.039$). Both approaches revealed higher variation within population as compared to among populations.

Table 9: Analysis of Molecular Variance (AMOVA) among and within four populations of Finger millet (E. coracana) with varying seed coat color

Source of	d.f	SS	MS	Variance	% of variation	P-value
Variation among populations	3	28.292	9.431	0.113	2%	P<0.039
Variation within populations	68	503.056	7.398	7.398	98%	
Total	**71**	**531.347**		**7.511**	**100%**	

d.f = Degree of freedom, SS = Sum of square, MS = Mean of square

6. DISCUSSION

6.1. ISSR polymorphism in finger millet diversity study

The range of loci and average number of loci reproduced in the present investigation were in comparison with earlier studies with random primers. Salimath *et al.* (1995) reported 26 % polymorphism in 17 finger millet genotypes with 6 ISSR primers, whereas Gupta *et al.* (2010) studied the genetic relatedness of three varieties of finger millet and reported 57 loci out of which 18 were polymorphic (31.58%) and 39 were monomorphic loci from 10 ISSR primers, with an average of 5.7 loci per primer.

In the present investigation, the details of polymorphism pattern of ISSR primers indicated that out of 45, 30 loci were observed to be polymorphic (66.67%). The extent of polymorphism was higher as compared to the earlier reports of Salimath *et al.* (1995) and Gupta *et al.* (2010) who reported 26.00, 31.58 percent polymorphic loci, respectively, with arbitrary primers in finger millet. The high percent polymorphism observed in this study could be due to inclusion of large number of finger millet accessions, and landraces. Similar results were reported by Shingane (2012) in foxtail millet, and Haftom *et al*., (2017) also found 77.78 % percentage of polymorphism on 80 genotypes of finger millet from Ethiopia, Zimbabwe and India using six ISSR primers.

Similarly, Fakrudin *et al*., (2004) got 6.04 loci on 32 finger millet genotypes using 45 average numbers of bands per primer using RAPD; Babu *et al*., (2006) reported 9.60 using 50 RAPD primers on 42 finger millet genotypes, and Kebere Bezaweletaw (2011) also found 8.2 using 15 RAPD primers. Moreover, the choice of appropriate primer motives in ISSR fingerprint is to detect high polymorphism and reveal relationship within and among populations. The abundance and distribution of SSRs in the genomes of finger millet could be another factor that determines the levels of polymorphism. In the present investigation, the landraces of finger millet with variable seed coat color showed higher number of fragments. This may be due to the more number of alleles accumulated in them from diverse parents.

The six ISSR primers chosen for this study amplified large number of loci, displaying 66.67% polymorphism with high gene diversity (h= 0.35) and Shannon's information index (I= 0.51). This is an evidence for the presence of high degree of variability among *E. coracana* accessions

having variable seed coat color in the study area. Among the four populations, Ahiforom, Mereb-Leke, Asgede-Tsimbla, and Laelay-Adyabo were found diversified geographically. The accessions red seed coat color and white seed coat color could not be further separated with each other which give an indication that they are genetically very similar. The possible reason for that both of the accessions have at least one common variety in their parentage or it is also possible that parents of both the accessions are genetically very similar.

6.2. Genetic relationship among finger millet genotypes

The cluster analysis based on un-weighted paired group method of arithmetic means (UPGMA) with 6 ISSR primers allowed the discrimination of the accessions used in this study. The dendrogram based on NJ similarity coefficient values calculated using ISSR marker data of 72 finger millet accessions with varying seed coat color clearly indicated moderate to high genetic diversity among the finger millet landrace accessions. The similarity coefficient for the genotypes varied from 0.64 to 1. The range of similarity coefficient was wider than the findings of Salimath *et al.* (1995) who reported similarity coefficient from 0.62 to 0.92 in 22 finger millet genotypes and Gupta *et al.* (2010) who observed values in the range of 0.68 to 0.90 in finger millet varieties. The wider range in similarity coefficient indicated large genetic diversity in materials studied which may be due to the use of more number of genotypes from landrace's, and diverse sources than that of previous reports.

The UPGMA based clustering grouped 72 finger millet genotypes into three major clusters. Cluster II had maximum number of genotypes (47) followed by cluster III (13) and I (12). Cluster I was sub-grouped into two sub-clusters, cluster II was sub-grouped into four sub-clusters, and cluster III sub-grouped into two sub-clusters. This is an indication that they are genetically very similar. The possible reason for that those landrace accessions have at least one common variety in their parentage or it is also possible that parents of both the landrace accessions are genetically very similar and the same geographical origin. Similar results were obtained in two African cultivar of Trichonella which were classified in same cluster by both RAPD and ISSR analysis (Dangi, *et al*., 2004). Salimath *et al.* (1995) grouped 22 finger millet genotypes into three clusters based on amplification pattern generated by 6 ISSR primers whereas Shingane (2012) grouped 44 foxtail millet genotypes into three major clusters based on 12 ISSR primers.

The independent grouping based on seed coat color landrace accessions indicated the differences in the genetic architecture of these genotypes. Cluster I comprised 12 genotypes. The similarity coefficient between any two genotypes in this cluster varied from 0.73 to 0.99 indicating similarity among the genotypes.

The present study was in agreement with the finding of Haftom *et al*., (2017). UPGMA tree showed a clear clustering of accessions on the basis of their regions of collection and respective populations, and the range of genetic similarity among the total accessions was in the range of 60-100%. Fakrudin *et al*. (2004) also found a clear apportionment of finger millet accessions in concordance with geographical origin using RAPD marker, while in contrast Kebre Bezawletaw (2011) reported no clear-cut clustering of accessions to their geographic origin. Though the accessions assessed in this study mainly represented landraces accessions having different seed coat color from four woredas of two Zones of Tigray region state of Ethiopia, the analysis of UPGMA tree showed a clear-cut pattern of variation in relation to geographical area, which could be due to the long history of domestication and cultivation of finger millet that might have resulted in the development of local landraces limited to a particular location coupled with limited gene flow where farmers save their seeds. The genetic structure observed in all the clustering analysis showed that there was moderate to low long distance gene flow but there is high short distance gene flow among populations of finger millet with varying seed coat color from the population of the study.

The ISSR primers differentiated the landrace accessions according to their respective genotypic makeup which shows their importance in differentiating the finger millet genotype at molecular level. These studies can be useful for DNA fingerprinting and molecular identification of finger millet in the future. The present investigation was in conformation with the earlier studies conducted by Salimath *et al.* (1995), Shingane (2012), Haftom *et al*., (2017), who reported the usefulness of ISSR markers for fingerprinting and to study phylogenetic analysis of finger millet genotypes and foxtail millet, respectively. Gupta *et al.* (2010) also emphasized on the usefulness of these markers for differentiating traditional and improved lines of finger millet with varying seed coat colors.

6.3. Genetic structure and classifying genetic diversity

The population genetic structure of a species reflects to the interactions among different processes during a long evolutionary history of species including shifts in distribution, habitat fragmentation and population isolation, mutation, genetic drift, reproductive biology, gene flow, and selection. Reproductive biology is likely to be particularly important in determining genetic structure of a given population (Hamrick and Godt, 1996).

In the present study, analysis of molecular variance (AMOVA) using six primers on four finger millet populations with variable seed coat color showed among population differentiation and within-population variation of, 2% and 98 %, respectively. The extent of variation was higher compared to earlier report of Dagnachew *et al.,* (2014), who reported 69.52% of the total allelic variation within populations and 30.48% among populations, respectively. This implies the presence of high gene flow among populations in different geographic locations the study area. High genetic variation within populations indicated that low genetic similarities exist among the individual plants sampled from a single population. Higher within population genetic variation might be due to high genetic exchange or gene flow, which actually has a more homogenizing effect on the genetic variation among populations by the dispersal of the seeds and seed exchange via market channels.

7. CONCLUSIONS

Seventy two finger millet landrace accessions were assessed with six ISSR primers. ISSR markers are useful to detect successfully genetic diversity and relationship at inter and intra population level of finger millet. High genetic variability was revealed within finger millet (*Eleusine coracana*) accessions. The assessed genetic diversity level varied within populations which might be due to difference in seed coat color, environmental conditions and human selection pressure. The high genetic variation within populations than among populations that was revealed by the AMOVA analysis shows the existence of high gene flow and low genetic differentiation among populations. The UPGMA cluster analysis indicated the grouping of accessions according to their geographical location. The UPGMA cluster analysis also revealed that the three varieties were grouped in three major clusters at the demarcation of 73% genetic similarity. Cluster I comprised a variety of black and red seed coat color from Ahiforom population and cluster II comprised intermixed populations for the three varieties, and the third cluster consisted of black and red from Mereb-leke population.

The genetic relatedness among three varieties of finger millet with different seed coat color has been investigated using six ISSR markers. The red seed coat color variety of finger millet is a hybrid, generated by crossing black and white varieties. The accessions, Ahiforom population and Mereb-leke population showed the lowest similarity. It is evident that higher genetic dissimilarity was observed between accessions from Ahiforom population and Mereb-leke suggesting that better opportunity to use accessions from these localities in hybridization programs. Since, hybrid vigor has a positive relation with genetic distance; hybridization between these two lines will provide progenies with greater variation at molecular level for selection of agronomically important traits. Therefore the finger millet accessions used in this study are genetically similar, but geographically diverse; rich genetic resource coupled with the generated information offers a good opportunity for undertaking further breeding activities through selection of the most diverse parents for finger millet yield improvements.

8. RECOMMENDATIONS

- This study was done using ISSR markers. Therefore, further study with different molecular markers such as SSRs and SNPs as well may be needed to confirm the result of the present study as a single molecular marker cannot fulfill all of the desirable properties of molecular markers.
- The most important attributes for introducing finger millet in the local market to generate income is based on seed coat color. Due to this, it is necessary to establish useful studies in finger millet with variable seed coat color at morphological or molecular level to increase production and productivity.
- This study has resulted in the development of markers that are an important genomic resource for finger millet. We recommend that the polymorphic loci be used to further advance genetic studies in finger millet.
- The variability among the finger millet accessions can be useful for selecting most diverse parents for use in developing finger millet populations' for mapping quantitative traits and studying their inheritance.
- More field research is needed to mine the available genetic resource of useful genes that could be used to enhance breeding of finger millet for farmer-preferred traits.

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10. APPENDICES

Appendix 1. DNA extraction protocol (Source; Borsch *et al.*, (2003)).

1. Pour CTAB solution (700 µl per sample) in a 15ml-tube and add 0.2 vol % Mercapto-ethanol (use fume hood!). Mercapto-ethanol is stored at 4.

2. Aliquot CTAB in 1.5 ml eppendorf-caps and warm in water bath up to 65 .

3. Weigh in 100 mg fresh leave material (50mg dry material) per sample. Pulverize thoroughly using a clean mortar and pestle. For fresh material add liquid nitrogen or quartz sand for dry material. First grind down slightly, then more powerful (cells have to be crashed). Use safety goggles!

4. Transfer the powder into an Eppendorf cap with warm CTAB solution immediately (use a new, clean spatula for each sample)

5. Add 700 µl of warm CTAB solution to the powdered sample (open the caps carefully), dissolve the powder and incubate the sample for 30 minutes at 65

6. Centrifuge for 5 minutes at 15000 rpm.

7. Transfer the supernatant (only clear liquid) in a new eppendorf-cap. Use blue pipette tips which are cut.

8. Add new CTAB solution (700 µl) to the tissue pellet and stir slightly with a new 1000 µl pipette tip, incubate 30 min at 65 . Step 6 and 7 are repeated. The same is carried out for a third extraction. Each fraction proceeds with step 9 and is treated separately.

9. Add 600 µl chloroform to the cap with supernatant and shake carefully a few times upside down. This chloroform step should be carried out immediately.

10. Shake the samples thoroughly by turning inversing the eppendorf caps for approximately 5 minutes. (Longer incubation is possible)

11. Centrifuge for 5 min at 15000 rpm.

12. Transfer the supernatant (only clear liquid) in a new Eppendorf-cap. Use blue pipette tips which are cut. Work carefully; do not transfer suspended matter (normally the chloroform is covered by a thin layer of fine sediment material). Chloroform has to be disposed of in a special waste bottle.

13. Repeat the chloroform extraction (step 9-12) to make sure that all impurities are removed, and then proceed with step 14.

14. Add cooled iso-propanol (4), approximately 2/3 of the solution volume. Shake carefully by inversing the eppendorff cap. In most cases DNA becomes visible as white threads. Freeze for more than 2 h at -20 . (BREAK POSSIBLE)

15. Centrifuge 10 min at 15000 rpm.

16. Aspirate liquid using yellow tips (without touching pellet!). If pellet is solid enough the larger part of the liquid may be poured out. (Alternatively add TE and proceed with qiagen kit)

17. Add 200 µl ethanol 70 % to the pellet. Rinse the inner cap surface by turning the cap.

18. Centrifuge for 10 min at 15000 rpm in a cooled centrifuge.

19. Aspirate ethanol using yellow tips. Dry the DNA-pellet at room temperature. (Usually 15 min are sufficient; after drying no liquid drops are to be seen)

20. Dissolve pellet in 100 µl TE (1x, p.a. grade) and store at 4°c. (BREAK POSSIBLE)

21. Add cooled 7.5 m nh4ac-solution (4°c, half of the solution volume). Mix carefully.

22. Add cool ethanol 100 % (double of the solution volume). Mix carefully. Freeze for more than 2 h at -20°c. (BREAK POSSIBLE)

23. Centrifuge 30 min at 15000 rpm. Aspirate fluid carefully.

24. Add 200 µl ethanol 70%. Rinse the inner cap surface by turning the cap.

25. Centrifuge 10 min. at 15000 rpm. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade)

26. Repeat steps 21 to 24 with 3 M NaAC solution (4°c, half the volume) then proceed with step

27. Centrifuge 10 min. at 15000 rpm. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade)

- cleaning the mortar and pestle:
- rinse the mortar and pestle with water
- clean the mortar and pestle in a 1:10 klorex-bath for 24 hours
- rinse with ddH2o
- Autoclave the mortar and pestle wrapped in aluminum foil at 134°c