ANALYSIS OF GENETIC VARIABILITY AMONG GERMPLASM OF FINGER MILLET BY USING ISSR MARKER

By

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A thesis submitted to the

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(AGRICULTURAL UNIVERSITY)

DIST. RATNAGIRI (MAHARASHTRA STATE), INDIA

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CERTIFICATE

This is to certify that the thesis entitled, **"Analysis of genetic variability among germplasm of finger millet by using ISSR marker"** submitted to the Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri, Maharashtra State, in the partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURE BIOTECHNOLOGY,** embodies the results of a piece of bona-fide research carried out by **Mr. KELKAR VIPUL GANESH** (Regd.No.0017) under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma or published in other form. All the assistance and help received during the course of investigation and the sources of literature have been duly acknowledged by him.

Place: Dapoli

Date :

(S. G. Bhave)
Chairman,
Advisory Committee
and
Research Guide

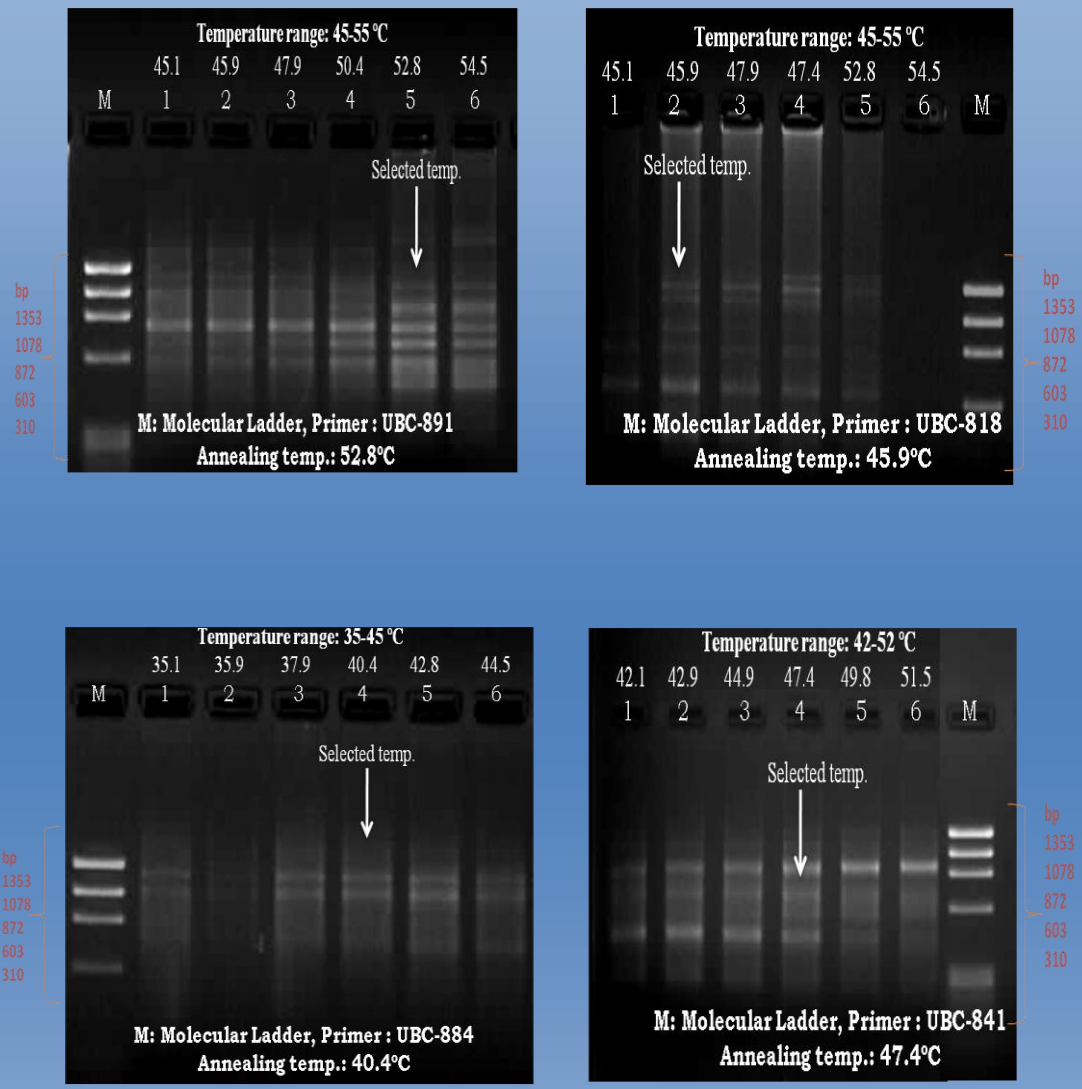

Plate : Gel photograph of Standardized Annealing temperature of ISSR Markers UBC-818, UBC-841, UBC-884 and UBC-891.

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"Knowledge is like a fruit.
When a fruit grows on a branch of a tree,
Its weight causes that branch to bend and bow.
Similarly when knowledge increases in a person,
It causes him to become humble and not proud and boastful."

The globe turns round and the time passes, by passing of time every beautiful thing come to an end. As the end of my post-graduation is in sight, a sudden realization makes me ponder over the last two years. It is indeed an opportunity to express sincere gratitude towards all who wished me success and helped me in my studies.

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We have two types of friends; friends for a reason and friends for season which help us in need, I avail this opportunity to thank my seniors ***Devendra, Parag, Anil, Moses*** *and* ***Shruti.****; my friends,* ***Kaustubh, Ashish, Parmeshwar, Sachin, Nikhil, Rahul, Ashutosh*** *and my loving junior* ***Babasaheb, Supriya M., Supriya B., Suvidha*** *and my very co-operative batch-mates* ***Ajinkya and Saurabh*** *I avail this opportunity to thank Ph. D. scholars, my seniors and my best friends* ***Navdeep Mhatre, Gaurish Swant, Somnath Jadhav, Aniruddha Khaire, Jyotsana Kshirsagar, and Vaishali Sapkal*** *for co-operation and lovely company during the M. Sc. degree Programme.*

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Finally thanking the Almighty for this wonderful life…

Date: *(V. G. Kelkar.)*

APPENDIX I

ABBREVIATIONS

°C	**:**	Degree Celsius
µl	**:**	Micro litre
AFLP	**:**	Amplified Fragment Length Polymorphism
Bp	**:**	Base Pairs
CIA	**:**	Chloroform-Isoamyl Alcohol
DNA	**:**	Deoxyribose Nucleic Acid
dNTPs	**:**	Deoxyribo Nucleoside tri-phosphate
dNTPs	**:**	Deoxy Nucleoside Triphosphates
EDTA	**:**	Ethylene Diamine Tetra Acetic Acid
et al.	**:**	And others
EtBr	**:**	Ethidium Bromide
FAO	**:**	Food and Agriculture Orgnization
Ha	**:**	Hectare
ICRISAT	**:**	International Crop Research Institute for the Semi-Arid Tropics
ISSR	**:**	Inter Simple Sequence Repeat
Kbp	**:**	Kilo base pairs
Mg	**:**	Miligram
$MgCl_2$	**:**	Magnesium Chloride

mM : Milimolar

MPa : Megapascal

MT : Metric tonnes

MVSP : Multi Variant Statistical Package

NaCl : Sodium Chloride

nm : Nano meter

OD : Optical Density

PAGE : Polyacrylamide Gel Electrophoresis

PCR : Polymarase Chain Reaction

PEG : Polyethylene Glycol

PIC : Polymorphic Information Content

Pm : Pico mole

PVP : Polyvinyl Pyrrolidone

RAPD : Random Amplified Polymorphic DNA

RFLPs : Restriction Fragment Length Polymorphism

RNA : Ribose Nucleic Acid

RNAse : Ribonuclease enzyme

Rpm : revolutions per minute

SDS : Sodium Dodecyl Sulphate

SSRs : Simple Sequence Repeates

TAE : Tris-Acetate EDTA

TE : Tris Buffer

T : Tonne

Tris HCL : Tris Hydrochloride

U : Unit

UPGMA : Unweighted Paired Group Method Using Arithmetic Averages

UV : Ultra Violet

Viz. : Namely

APPENDIX II

Composition of Chemicals

CIA (25 ml)

Chloroform	24 ml
Isoamyl alcohol	1 ml

Ethidium bromide (10 ml)

EtBr	0.01 g
Distilled water	10 ml
Stored at 4^0C	

6X Loading dye (50 ml)

0.25% Bromophenol blue	125 mg
40% sucrose in water	20 g
Stored at 4^0C	

5% Sarcosyl (10ml)

Sarcosyl	0.5g
Distilled water	10 ml

1X TE buffer (10 ml)

10X TE	1ml
Sterile water	9ml

50X TAE (1 Litre)

Tris Base	242g
Glacial acetic acid	57.1ml
0.25 M EDTA (pH 8.0)	200ml
Final volume	1000ml

CHPATER I

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) Gaertn.) 2n=36, is a poor man's crop, originated in Ethiopia (Vavilov 1951). It belongs to the tribe Chloridae of the family Poaceae. Finger millet was domesticated about 5000 years ago in eastern Africa (possibly Ethiopia) and introduced into India, 3000 years ago (Hilu *et al.*, 1979). The closest wild relative of finger millet is *E. coracana* subsp. *africana* (Hilu *et al.*, 1979) which is native of Africa.

Finger millet is the primary food for millions in dry lands of east and central Africa and southern India. (Prabhu *et. al.,* 2013). It is cultivated for human consumption in Sub-Saharan Africa and South Asia.

Finger millet commonly referred as ragi or nagali or nachani ranks third in importance among millets in India. Its cultivation is more widespread compared to other millets and accounts for 8% of the area and 11% production of all the millets cultivation in the world (Gupta *et. al.,* 2010). It is also considered a helpful famine crop as it is easily stored for lean years (FAO, 2012).

The global annual planting area of finger millet is estimated at around 4-4.5 million hectare with a total production of 5 million tons of grain, of which India alone produces about 1.6 million tones and Africa about 1.5 million tones. Finger millet contributes nearly 40 per cent of small millets in India. It is widely grown in Karnataka, Tamil Nadu, Andhra Pradesh, Orissa, Bihar, Gujarat, Maharashtra and in the hilly regions of Uttar Pradesh and Himachal Pradesh (Anon; 2012-13).

The area and production of ragi in India in *kharif* 2012-13 was 1.12 million hectare 1.57 million tones, respectively. In Maharashtra, area under finger millet was 166.8 thousand hectare with production of 170.2 thousand tones. In the Konkan region of Maharashtra it is cultivated in the area of 38.488 thousand hectare comprising Raigad, Thane, Palghar, Sindhudurga and Ratnagiri district with production of 41.136 thousand tones (agrimaha.com 2015). However the productivity of finger millet in these districts is low (1167 kg/ha) (Anon; 2012-13).

The grain is readily digestible, highly nutritious and versatile. It can be cooked like rice, ground to make porridge or flour or used to make cakes. Sprouted grains are recommended for infants and elderly people. Finger millet is also used to make liquor ("*arake*" or "*areki*" in Ethiopia) and beer, which yields by-products used for livestock feeding (FAO, 2012). Like other cereal grains, finger millet is an energy feed valuable for its high carbohydrate content (80%). The protein content (7-10%) is often slightly lower and its fiber content is higher (crude fiber 4-9% of dry matter) than that of maize grain and pearl millet. Ragi is the potential replacement for maize after pearl millet in areas where the three crops are available.

Ragi is commercially gaining importance as it has a great potential for industrial use especially weaning foods because it is a rich source of Iron (380 ppm) and Calcium (275 ppm). The grain of finger millet has a fine aroma when cooked or roasted and is known to have many health-promoting qualities. The crop provides food grain as well as straw which are valued animal feed, especially in the rainfed areas. Despite of so many merits,

finger millet has remained neglected crop compared to other cereals such as maize, rice and wheat.

During the past decade, phenotypical characteristics have been used for classification and identification of species or varieties. Taking into account the utility, the conservation of genetic diversity and building up of nuclear base populations are essential for the improvement of cereal crops. The most important role of conservation is to preserve the genetic variation and evolutionary process in viable populations of ecologically and commercially viable genotypes in order to prevent potential extinction. The molecular approach for identification of plant genotypes seems to be more effective than traditional morphological markers because it allows direct access to the hereditary material and makes it possible to understand the relationships between plants. (Das *et. al.*, 2009).

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. Several statistical techniques are available for the analysis of genetic diversity using DNA fingerprinting data (Prabhu and Ganesan, 2013). Assessment of genetic diversity using DNA markers is one of the key tools of crop improvement and germplasm conservation. Several reports are available assessing the genetic diversity in finger millet using DNA based molecular markers namely ISSR (Dellaporta *et. al.*, 1983) and RAPD (Fakrudin *et. al.*, 2004, Salimath *et. al.*, 1995).

The genetic improvement of crop depends on the amount of genetic variability present in the population. Genetic diversity is normally assessed by common morphological traits. However, such traits are affected by effects of environment, development

stage of the plant and the type of plant material and also it require several replications to establish the genotypic contributions (Prabhu and Ganesan, 2013). Hence, there is a need to go 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. Molecular markers have also provide a powerful tool for breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited characters. Among the different molecular markers, the ISSR technique is a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species (Moreno *et. al.*, 1998).

Till now very little efforts have been made to assess the genetic variability among finger millet genotypes from Indian sub-continent at molecular level. There is little information on the extent of genetic variability at molecular level among finger millet accessions adapted to various States of peninsular region of India (Gupta *et al.* 2012). The information on genetic variability and component analysis can be of great help in formulating appropriate breeding strategy for genetic upgradation of ragi (Lal *et al.* 1996).

Thus, The present study was undertaken with the objective to analyze the genetic variability among the germplasm of finger millet through ISSR marker.

CHAPTER II

REVIEW OF LITERATURE

Finger millet is highly self-pollinated allotetraploid (2n = 36) derived from the wild tetraploid progenitor *E. coracana* subsp. *africana*. At present 55 to 60 per cent of the finger millet crop is grown in southern and Central Africa, and remaining is produced in India (Rao and Mushonga, 1985).

There is growing evidence and reorganization that a staggering burden of disease and death is posed by chronic micronutrient deficiencies even in seemingly healthy people. Deficiency of vitamin A, iron and zinc, are major contributing causes to several health hazards in children and adult people. Among trace elements iron and zinc in edible parts of plants are much lower than the required level for human nutrition. Attempts have been made to improve iron status in some crops, but as such very limited attempts made with regard to zinc. Nearly 49 per cent of global population does not meet their recommended dose, making zinc deficiency. The processing of finger millet (malting, grinding, sprouting, and puffing) reduces antinutritional factors, there by resulting in better availability of micronutrients (Rao Sankara and Deosthale, 1983).

In case of finger millet besides several phenotypic or morphological descriptors, various molecular markers have been utilized for characterizing the germplasms. (Gupta *et al.* 2010) 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. Several statistical techniques are available for

the analysis of genetic diversity using DNA fingerprinting data. A brief review of literature related to the present study entitled "Analysis of genetic variability among germplasm of finger millet by using ISSR marker" is discussed in this chapter under different headings.

2.1 Morphological markers

Although morphological markers are technically simple, they have several limitations. Discrete morphological traits, which show high heritability, are limited in number, each being governed by a few genes and hence, cover only a small portion of the genome. Quantitative traits are influenced by environmental factors, implying that these traits show continuous variation. This results in low heritability and high genotype by environment interactions that make it difficult to determine genetic variation accurately.

Doebley (1989) concluded that the traditionally, classification of the various subgenera, species and subspecies is based primarily on morphological attributes. However, these traits may not be significantly distinct and usually require growing plants to maturity prior to identification. Moreover, morphological characters may be unstable due to environmental influences. Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits. Accessions of morphologically different characters including shape of seeds, seed coat colour, etc., are very close according to the dendrogram constructed based on the presence or absence of amplified DNA fragments of a particular size. The discrepancy

between molecular genetic diversity and morphological diversity has been well documented.

Duminil and Michele, (2009) reported that field species recognition and sampling are generally based on morphological characters, but they can either fail to discriminate species and mask the presence of cryptic species or discriminate different species while in reality there is only one. To overcome this problem it is common to compare clusters obtained on the basis of the observed polymorphism of both characters, and to analyse their agreement.

Kumar *et al.*, (2010) recorded the data on 10 quantitative traits *viz.* days to 50 % flowering, days to maturity, plant height, number of tillers/plant, productive tillers/plant, number of fingers/ear, total fingers/plant, biological yield, grain yield and harvest index were collected from a random sample of 10 plants for each genotype. On the basis of non-hierarchical Euclidean cluster analysis, all the 140 genotypes of the present study were grouped into ten non-overlapping clusters.

Goitseone Malambane *et al.*, (2010) evaluated thirty-five finger millet accession in the dry season 2010/11 and the rainy season 2011. Combined analyses showed significant differences among seasons, genotype and genotype by season interactions for yield per plot, 1000 seed weight, finger length, plant height and days to flowering. Season contributed to a large proportion of variations on yield per plot (97.4 %), 1000-seed weight (93.5 %), finger length (62.8%), plant height (94.6%) and days to flowering (78%). However, variations due to genotype were (38.5%) for finger number, finger width (41.8%) and days to flowering (16.8%). Accession IE 3618 showed low variation in

yield for the two seasons while IE 4565 had the highest variations when evaluated in the two seasons for yield per plot. Because of high yield and low variation, IE 3618 is promising for production in both the dry season and the rainy season.

Gupta *et al.*, (2010) carried out study based on seed colors as phenotypic descriptor attempt has been made to analyze 36 genotypes of finger millet [*Eleusine coracana* (L.) Gaertn] and correlate with protein and calcium contents.

Nandini *et al.*, (2010) reported that improvement of economic characters like yield through selection is conditioned by the nature and magnitude of variability existing in such populations. However, the phenotypic expression of complex character like yield is a combination of genotype, environment and their interaction. This indicates the need for partition of overall variability into heritable and non-heritable components with the help of appropriate statistical techniques. The results of this investigation carried out in F_2 generation of four crosses of finger millet to quantify the variability created along with heritability and genetic advances for important yield and attributing characters.

Dhanalakshmi *et al.*, (2013) evaluated 622 core finger millet germplasm accessions for six quantitative characters. The results revealed substantial genetic variability for all the traits investigated. The contribution of genetic variability was greater in relation to environmental variability towards total variability for all the investigated traits among the germplasm accessions. These results are discussed in relation to the development of strategic genetic material for localizing genes controlling

economic traits and designing suitable breeding and selection strategies for genetic improvement of finger millet.

Karad and Patil (2013) studied a set of sixty-five finger millet (*Eleusine coracana* L.) accessions and they were screened for twelve morphological characteristics to study nature and magnitude of genetic divergence using Mahalanobis D2 statistics during *kharif* 2009 and *kharif* 2010. Wide range of variation was observed for all the twelve characters under study. The analysis of variance exhibited significant difference among the genotypes for all the characters indicating substantial degree of variability.

Prabhu and Ganeshan (2013) concluded that genetic diversity is normally assessed by common morphological traits. However, such traits are affected by effects of environment, development stage of the plant and the type of plant material and also it require several replications to establish the genotypic contributions. Hence, there is a need to go in 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.

Umar and Kwon-Ndung (2014) studied was conducted to assess the phenotypic variation/diversity of 10 germplasm accessions of Finger millet (*Eleusine coracana* L.) from diverse locations in the geographic region of Northern Nigeria during the 2008, 2009 and 2010 cropping seasons. Randomised Complete Block Design (RCBD) was used for the study and field data were analysed based on phenotypic characters. Phenotypes were found to express significant diversity for plant height, 1000 seed weight, leaf length and number of tillers. The results were analysed using ANOVA model and showed that plant height in

accession Ex-Kwi was significantly different from all the other nine accessions while the highest leaf length which was recorded in Ex-Riyom was significantly different ($p<0.05$) from accession Ex-Dantse. Similarly, significant variations were observed in the number and length of fingers, and 1000 seed weight across all the accessions. Cluster analyses revealed six distinct groups, with one landrace forming an independent colony. Our results suggest a high phenotypic variability, which could exist among the selected morphological traits

2.2 Biochemical markers

The most commonly used protein markers are isozymes. Functional enzymes that are multimeric in nature normally exist in different molecular forms. It has been recognized that, while retaining substrate specificity, these forms (isozymes) are distinguishable based on electrophoretic mobility (Markert and Muller, 1959). Polymorphic isozymes have been used for tagging genes for disease resistance. Isozymes are enzymes that share a common substrate but differ in electrophoretic mobility. Isozymes have largely been used in cowpea improvement programmes with emphasis on populations, taxonomy, genetic relationship and diversity studies.

Uma *et al.* (1995) studied synthesis of stress induced proteins in two contrasting genotypes. Marked differences were found among 28 finger millet genotypes (*Eleusine corcana Gartn.*) in aquired tolerance to osmotic stress as accessed by the recovery of root growth from severe stress (1.2 MPa polyethylene glycol or 400 mM NaCl). However, these differences in tolerance were observed only when the seedlings were subjected to a

proceeding mild induction stress (0.6 MPa PEG or 200 mM NaCl).

Jayprakash *et al.*, (1998) reported the extent of genetic variability in finger millet the level of expression of lea2 and lea3 under stress in finger millet and rice seedlings. Plants have developed different adaptive mechanisms to withstand abiotic stress conditions such as high or low temperature, salinity and drought. Many of these adaptive mechanisms are a consequence of stress perception and are likely to be mediated through stress induced expression of specific genes. This stress induced gene expression leads to the synthesis of specific stress responsive proteins, which may impart tolerance. Stress induced genes are many and diverse.

2.3. Molecular or DNA based markers

Molecular markers (DNA markers) are developed to overcome limitations of morphological data although it does not mean that any of the biochemical or molecular techniques or both replace morphological marker. Molecular markers have great potential for use in quality control in breeding programme. Presently, the term molecular marker invariably points to DNA based markers using a variety of techniques to assay variation at the DNA level. There are two classes of these markers, *viz.,* those based on DNA-DNA hybridization between a labelled DNA clone (probe) and total genomic DNA and the others employ PCR amplification.

Choudhary *et al.*, (2008) reported that the application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar

identification and characterization as well as determination of population diversity in many plant species.

Sinha and Pande (2010) reported that genetic fingerprinting or DNA profiling is the technique invented by Sir Alec Jeffreys and is used to distinguish between individuals of the same species using only samples of their DNA.

Naga *et al.* (2012) reported that in modern studies of plant genetics and breeding, molecular markers have become important and efficient tools for genetic diversity assessment, QTL and/or gene mapping, variety protection, and marker-assisted selection.

Prabhu and Ganeshan (2013) reported that 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.

2.3.1. Hybridization based markers (RFLPs)

RFLP analysis is now a standard, well-tested procedure for estimation of genetic diversity. It has been used in thousands of studies in a variety of species. RFLP markers have been used extensively for genome mapping and for analysis of genetic diversity. They have also been used in plant breeding programs for marker-assisted selection. RFLP markers tend to be inherited as simple Mendelian codominant alleles, and are not influenced in any way by environmental factors. Therefore, their heritabilities are invariably 100%. There are three major advantages to RFLP analysis over protein markers. First, the

number of polymorphic markers that can be generated is virtually limitless. No laboratory has ever exhausted the supply of potential RFLPs. Second, the procedure is the same for every marker, so once a laboratory is set up for RFLP analysis, no major changes need to be made for analyzing different markers. Third, the level of polymorphism is greater in RFLPs than in protein markers.

Salimath *et al.*, (1995) experimented with three different DNA marker techniques, *viz.,* RFLP (8 probe-3-enzyme combination), RAPD (18 primers) and ISSR (6 primers) and analyzed the diversity of 22 accessions belong to 5 species of *Eleusine*. The results revealed 14, 10 and 26 per cent polymorphisms in 17 accessions of *E. coracana* from Africa and Asia and very low level of DNA sequence variability in finger millet.They suggested that the ISSR marker was good as compared to RFLP and RAPD in terms of the quantity and quality of data output.

Muza *et al.*, (1995) reported diversity of 26 germplasm lines of finger millet from Africa and India based on the southern blot hybridization patterns obtained with maize and sorghum mitochondrial cloned gene probes with five restriction endonuclease enzymes. A total of 20 enzyme/probe combinations observed a low level of polymorphism/ with identical RFLP banding patterns in 23 of the 26 lines. Based on this data, 26 germplasm lines were classified into 3 cluster groups**.**

Salimath *et al.*, (1995) reported molecular diversity of 20 finger millet accessions by using RFLP markers. Sixteen isozyme

loci and 15 RFLP loci showed uniformity among the accessions indicating less genetic diversity among the accessions.

2.3.2. PCR based markers

Wang *et al.* (2005) made an attempt to identify the transferability of 210 SSR markers from major cereal crops (wheat, rice, maize, and sorghum) to *E. coracana* and *more* than half (57%) of the SSR primers screened, generated reproducible cross-species or cross-genus amplicons. They described that the transfer rate of SSR markers was correlated with the phylogenetic relationship or genetic relatedness.

Kumar *et al.*, (2006) carried out study on the inter-simple sequence repeats (ISSRs), a technique based on DNA polymerase chain reaction (PCR) method, is used to examine the molecular polymorphisms in *Pennisetum glaucum.* ISSR analysis was carried out for the 12 pearl millet genotypes using five ISSR primers. The results indicated each genotype generated unique banding profiles.

Babu *et al.*, (2007) reported the diversity of 32 finger millet genotypes, using 50 RAPD markers and reported a total 529 loci of which 479 loci (91%) were polymorphic and informative to differentiate the accessions and do cluster analysis. They grouped the 32 finger millet accessions into two major clusters, and genotypes GEC 182 and CO 12 were distantly related with a low similarity index of 0.315 and also differed considerably in days to flowering and grain weight. Diversity of 30 finger millet genotypes by using 13 RAPD primers was reported by Das *et al.,* (2009). A total of 124 distinct DNA fragments ranging from 300 to 3000 bp were reported. The genetic similarity and cluster analysis based on similarity coefficient indicated two major

clusters, first major cluster had one genotype and a second majorcluster contained 29 genotypes.

Dida *et al.*, (2007) developed a first genetic map of finger millet by using RFLP,AFLP, EST and SSR markers. The map span was 721 cM on the A genome and 787 cM on the B genome and cover all 18 finger millet chromosomes. They developed a set of 82 SSR markers specific for finger millet by small-insert genomic libraries generated using methylation-sensitive restriction enzymes and among them, 31SSRs were mapped. Comparative analysis of this map with rice genetic map (International rice genome sequencing project, 2005) was a novel attempt reported high level of conserved co-linearity between the finger millet and rice genomic.

Varshney *et al.*, (2007) concluded that characterization of genetic variation within natural populations and among breeding lines is crucial for effective conservation and exploitation of genetic resources for crop improvement programmes. Molecular markers have proven useful for assessment of genetic variation in germplasm collections. Evaluation of germplam with GMMs might enhance the role of genetic markers by assaying the variation in transcribed and known function genes, although there may be a higher probability of bias owing to selection.

Yadav *et al.*, (2007) carried out study on genetic diversity and observed relationships among 20 Indian pearl millet [*Pennisetum glaucum* (L.) genotypes representing the commercially important hybrids, cytoplasmic male sterile (CMS) lines, restorer (res) lines and open pollinated varieties (opv) by using inter simple sequence repeat (ISSR) markers. A total of 349 reproducible bands (73 monomorphic, 276 polymorphic)

were detected using 30 UBC 9 ISSR primers with polymorphism percentage of 79.1%. Forty-five of the polymorphic bands were in fact the unique bands that were present or absent exclusively in only one of the 20 genotypes. A set of two or any five ISSR-PCR assays were sufficient to clearly distinguish among the 20 genotypes.

Agarwal *et al.*, (2008) carried out detection and analysis of genetic variation to understand the molecular basis of various biological phenomena in plants. Since the entire plant kingdom cannot be covered under sequencing projects, molecular markers and their correlation to phenotypes provide us with requisite landmarks for elucidation of genetic variation. Recently, Dida *et al.* (2008) reported the population structure of 79 finger millet accessions with 45 SSR markers and identified significant difference of plant architecture and yield in Asian and African subpopulation.

Das *et al.*, (2009) studied on Molecular characterisation of the 15 early duration finger millet (*Eleusine coracana* G) genotypes through RAPD markers. Twenty-five decamer primers were used for initial screening. Of these nine primers (OPA4, OPA13, OPA16, OPC12, OPC18, OPD8, OPN7, OPN15 and OPN16) showed polymorphic banding pattern for the genotypes. Total number of bands produced ranged from 2 to 9 bands per primer. The nine primers produced 60 amplification products, of which 9 were monomorphic and 51 were polymorphic. The primers OPA4, OPC18 and OPN15 produced a greater number of polymorphic bands than OPA16 and OPN16.

Das and Misra (2010) studied genetic diversity among fifteen finger millet genotypes using RAPD markers. Out of the

initial twenty-five random primers, nine RAPD primers were found to be highly reproducible and produced a total of 60 loci of which 51 loci were polymorphic.

Gupta *et.al.*, (2010) studied the genetic relatedness of three varieties of finger millet (*Eleusine coracana* L.) with varying seed coat color namely PRM-1(Brown), PRM-701(White) and PRM-801 (Golden) with 10 RAPD and 10 ISSR markers. Molecular parameters *viz.*, total number of bands, average polymorphic bands, average percent polymorphism, average polymorphic information content (PIC) and average expected gene diversity (Hi) generated from RAPD and ISSR markers has been compared. The RAPD profiling of these varieties with 10 random primers generated 86 loci with 49 polymorphic and 37 monomorphic loci. The molecular characterization of these varieties using 10 ISSR markers generated 57 loci with 18 polymorphic and 39 monomorphic loci. The RAPD marker with 8.5 loci per primer was found to be better than ISSR marker showing an average of 5.7 loci per primer in the present study.

Kumari and Pande (2010) concluded that germplasm identification and characterization is an important link between conservation and utilization of plant genetic resources. The study was conducted to characterize the genetic diversity using twelve germplasm of finger millet including two of the same variety (VL-149) but from different regions. Three replica of each germplasm was amplified using seventeen random primers. A total of 113 distinct fragments ranging from 117 bp to 2621 bp were amplified. Of these, 70 (61.9%) were found to be polymorphic. The lowest and highest polymorphisms were obtained within individuals belonging to genotypes OUAT-2 and

VL-324. Both white seeded germplasms, showed maximum closeness. The study helped in identifying the germplasm in a quick and reproducible manner and studying their relatedness.

Panwar *et al.*, (2010) studied genetic relationships among 52 *Eleusine coracana* (finger millet) genotypes collected from different districts of Uttarakhand and were investigated by using randomly amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and cytochrome P450 gene based markers.

Alhajturki *et al.*, (2011) reported Inter Simple Sequence Repeat (ISSR) markers and were used to assess the genetic diversity of 10 sorghum varieties, nine of Indians and one of Syrian origins. Out of 20 ISSR primers screened, 9 primers were selected for their polymorphic and repeatable fragments.

Bezaweletaw (2011) evaluated sixty-six finger millet accessions composed of 64 landraces and two improved varieties using RAPD markers to study the genetic diversity in finger millet landraces. The RAPD analysis was executed using 15 primers, which were screened based on their effectiveness to discriminate among the accessions. Among the 123 RAPD fragments amplified, 89 (72.35%) were polymorphic. The polymorphic information content (PIC) ranged from 0 to 0.50 with heterogeneous distribution and about 23% of the markers with a high discrimination power of ≥ 0.30. The 66 accessions were grouped into nine clusters at similarity index of approximately 0.83; however, there was no clear-cut separation among finger millet accessions in relation to the origin of their respective region. The result of the present study revealed the existence of ample variability in finger millet landraces that could be employed in the genetic improvement.

Kumari *et al.*, (2011) analyzed a set of 125 foxtail millet accessions selected from 11 different agro ecological regions of India and analyzed using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) marker techniques. A total of 146 (115 RAPD and 31 ISSR) scorable fragments were generated with 16 RAPD and four ISSR primers.

Gupta *et al.*, (2012) concluded that the ISSR technique is a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species, including fruit trees. ISSR technique permits the detection of polymorphisms in microsatellites and inters microsatellites loci without previous knowledge of the DNA sequence. The technique involves the use of a single primer composed by a microsatellite sequence plus a short arbitrary sequence (anchor) which target a subset of 'simple sequence repeats' (SSRs) or microsatellites and amplify the region between two closely spaced and oppositely oriented SSRs . ISSRs have been used in assessing genetic relationships among various accessions of different species.

Arya *et al.*, (2013) addressed genetic diversity in finger millet by using different molecular markers (Salimath *et al.* 1995; Fakrudin *et al.* 2004; Babu *et al.* 2007; Dida *et al.* 2008; Panwar *et al.* 2010), but these studies are limited and more and more germplasm needs to be characterized at molecular level for identification of diverse germplasm lines/subpopulations to be used for crop improvement.

Hemaprabha *et al.* (2013) carried out study on Random amplified polymorphic DNA (RAPD) analysis and inter-simple sequence repeats (ISSR) primers to determine the occurrence

and extent of variation in rice (*Oryza sativa* L.) plants regenerated from anther culture. Genetic diversity among 27 regenerants of the cross CO43/Nootripathu and their parents were assessed using 25 RAPD primers and 19 ISSR primers. RAPD primers used in the study produced 285 polymorphic markers (81.65% polymorphism) and ISSR primers produced 201 polymorphic markers (79.37% polymorphism).

Paul and Panneerselvam (2013) carried out experiment with twenty five RAPD and ten ISSR primers for PCR amplification. Among those, RAPD primers generated 80 amplification products of which 22 were polymorphic and the ISSR markers produced 20 amplification products, out of which 7 were polymorphic. ISSR primers produced 35% polymorphic bands between the land races of *Setaria italica* when compared to 27.5% by RAPD analysis. ISSR marker systems were found to be more accurate for the genetic diversity studies in the land races of *Setaria italica* when compared to RAPD.

Prabhu and Ganeshan (2013) carried out an investigation on a collection of forty ragi genotypes to study the genetic diversity using ten SSR and 30 ISSR markers. There were successful amplification SSR and ISSR markers in ragi. Of the ten SSR primers six showed polymorphism, the number of alleles produced by SSR primers ranged from two to five with an average of 2. Out of the thirty ISSR primers used for molecular analysis, twenty primers showed polymorphism among forty ragi genotypes. The twenty polymorphic primers generated a total of 101 alleles. The number of alleles produced by different primers ranged between three and ten with an average of 5.05 alleles per primer. The percentage of amplification ranged from 33 (ISSR 20)

to 85 (ISSR 29). Forty genotypes grouped into thirteen clusters (SSR) and five clusters (ISSR), respectively in UPGMA analysis.

Karad *et al.*, (2013) reported among various technique of molecular approach, RAPD (random amplified polymorphic DNA) is one of the important technique use to access the germplasm diversity. The present investigation was carried out to investigate the genetic diversity of selected finger millet genotypes through the use of RAPD markers.

Ramadoss (2014) studied 83 accessions of finger millet obtained from ICRISAT was genotyped using 31 polymorphic simple sequence repeats (SSRs) identified from transcript assemblies. A total of 152 alleles were generated by these 31 SSRs, with a mean of 4.9 alleles per locus and mean polymorphism information content (PIC) of 0.49 Cluster analysis of these accessions showed considerable genetic variation in the varieties from different geographical origins. Identified polymorphic SSRs can be used to further expand the linkage map of finger millet.

Dagnachew *et al.*, (2014) conducted experiment with 72 accessions, sampled from five major species, *E. coracana* (including both *E. coracana* subsp. *coracana* and *E. coracana* subsp. *africana), E. intermedia, E. indica, E. multiflora and E. floccifolia* were analysed for genetic variation and inter-relationships using 20 microsatellite markers. All the SSR markers displayed high genetic polymorphism, with polymorphic information content ranging from 0.46 (UGEP110) to 0.91 (UGEP66). A total of 286 alleles were observed with an average of 14.3 alleles per locus. Classic F-statistics revealed the highest

intra-specific polymorphism recorded for *E. africana* (32.45%), followed by *E. coracana* (16.83%)

Yadav *et al.*, (2014) conducted experiment with 146 simple sequence repeat (SSR) primers were designed and evaluated for cross-transferability across a panel of nine grass species including finger millet. The average transferability of SSR markers from sorghum to other grasses was highest (73.2 %) followed by rice (63.4 %) with an overall average of 68.3 % which establishes the importance of these major crops as a useful resource of genomic information for minor crops. The transfer rate of SSR markers was also correlated with the phylogenetic relationship (or genetic relatedness) of the species. Primers with successful amplification in finger millet were further used to screen for polymorphism across a set of high and low calcium containing genotypes.

Garg Rashmi *et al.*, (2015) analyzed Inter Simple Sequence Repeat (ISSR) markers to assess the genetic relationship among 27 finger millet genotypes. The number of bands produced by different markers was ranging from 2 to 8. PIC value and Rp value were ranging from 0.143 to 0.983 and 0.52 to 11.56, respectively. The genetic similarity matrix varied from 0.43 to 0.98 in these genotypes. Dendogram analysis revealed formation of two major groups, one with five subgroups compris-ing 10 genotypes and the other one consisted of 17 genotypes in five subgroups.. The results re-vealed that ISSR markers could be efficiently used to quickly access the genetic variation available in the finger millet genotypes.

Ramakrishnan *et al.*, (2016) evaluated the genetic variation and population structure in Indian and non-Indian genotypes of

finger millet using 87 genomic SSR primers. The 128 finger millet genotypes were collected and genomic DNA was isolated. Eighty-seven genomic SSR primers with 60–70 % GC contents were used for PCR analysis of 128 finger millet genotypes. The mean major allele frequency was 0.92; the means of polymorphic alleles were 2.13 per primer and 1.45 per genotype; the average polymorphism was 59.94 % per primer and average PIC value was 0.44 per primer. Indian genotypes produced an additional 0.21 allele than non-Indian genotypes. Gene diversity was in the range from 0.02 to 0.35. The average heterozygosity was 0.11, close to 100 % homozygosity. The highest inbreeding coefficient was observed with SSR marker UGEP67. The Jaccard's similarity coefficient value ranged from 0.011 to 0.836. The highest similarity value was 0.836 between genotypes DPI009-04 and GPU-45.

CHAPTER III
MATERIAL AND METHODS

The detailed information regarding the material used and methods followed during the course of the present investigation is mentioned in this chapter. The investigations were carried out in the laboratory of Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri (M.S.) during the academic year 2014-2016.

3.1 Genetic Material:

In the present investigation 40 germplasm including variety of finger millet obtained from Department of Agriculture Botany, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. The list of germplasm are given in Table 1.

Table1: Details of germplasm used in the study.

Sr. No.	Name of Germplasm	Sr. No.	Name of Germplasm
1.	Nagali- 35	21	GPU- 69
2.	Nagali- 52	22	IGPSM – 10
3.	Nagali- 55	23	IGPSM-18
4.	Nagali- 56	24	OEB – 54
5.	Nagali- 61	25	OEB – 265
6.	Nagali- 62	26	L – 5
7.	Nagali- 66	27	L - 481
8.	Nagali- 67	28	VL - 149
9.	Nagali- 69	29	VL - 324
10.	Nagali- 2RJ	30	VR - 708
11.	Dapoli- 1	31	VR -762
12.	Dapli Safed	32	PR - 202
13.	Vakavali -02	33	PR - 1044
14.	Kolhapur	34	MR - 06
15.	GPU- 28	35	GSIS - 01
16.	GPU- 45	36	GOA - 712
17.	GPU- 48	37	PNV - 5
18.	GPU- 65	38	ACCR - 33
19.	GPU- 66	39	KOPN - 235
20.	GPU- 67	40	KMR - 204

3.2 Laboratory Resources and Techniques

The laboratory work was done in the laboratory of Plant Biotechnology Centre, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth Dapoli, Dist- Ratnagiri (M.S.).

3.2.1 Extraction of genomic DNA

3.2.1.1 Plant material

For the present experimental study, all 40 germplasm of finger millet were sown in the pots and kept in greenhouse. The leaf samples were collected from 10 days old seedlings for the extraction of genomic DNA.

3.2.1.2 Stock solutions prepared

The various solutions, buffers and its concentration used for extraction of the DNA are mentioned as under.

a) Extraction buffer

Table 2: Extraction buffer stock solutions:

Sr. No.	Chemicals	Quantity required for 100 ml. (g)
1.	200 mM Tris-HCL	2.423
2.	25 mM EDTA	0.831
3.	250 mM NaCl	1.461

Table 3: Composition of extraction buffer:

Sr. No.	Chemicals	Quantity required for 10 ml of EB
1.	0.5M glucose	900 mg
2.	0.5% SDS	50 mg
3.	3% PVP	300 mg
4.	0.4% Sodium bisulphate	40 mg
5.	5% Sarcosyl	500 µl

b) Chloroform Isoamyl alcohol mixture (24:1)

c) 100% Chilled Isopropanol

d) 70% Ethyl alcohol

e) 1X TE Buffer

3.2.1.3 Procedure for extraction of genomic DNA

The DNA was isolated by following the protocol of Doyle and Doyle (1990) i.e. Rapid method with slight modifications of buffer composition and concentration. The young newly emerged leaves were collected and sterilized with 70% ethanol to avoid the contamination. The extraction of genomic DNA was done using the following protocol:

1. Leaf tissue (100 mg) was collected in a 1.5 ml eppendorf tube which leads to ensure uniform size of sample.
2. Collected tissue was macerated by micro pestle at room temperature without buffer for 15 sec.
3. Extraction buffer (500 µl) was added and leaf tissue macerated gently for few seconds and kept in hot water bath for 45 minutes at 65^0C.
4. The sample was cooled down at room temperature and centrifuged at 8000 rpm, at 4^0C for 10 minutes.
5. Aqueous layer was transferred to fresh eppendorf tube and 200 µl of C:IA (24:1) was added and mixed by gentle inversion for 5-6 times. The contents were then centrifuged at 8000 rpm, at 4^0C for 10 minutes.
6. Supernatant was mixed with 2/3 of chilled isopropanol and incubated at –20^0C overnight.

7. On the next day, the solution was centrifuged at 8000 rpm, at 4^0C for 10 minutes and pellet was collected.
8. Pellet was washed with 100 µl of 70 per cent ethanol followed by centrifugation at 8000 rpm, at 4^0C for 10 minutes.
9. Pellet was dried and re-suspended in 50 µl of 1x TE buffer.
10. Incubated at 37°C in a water bath for 30 minutes and stored at -20°C till further use.

3.2.2 DNA Purification

Purification of DNA was done to remove RNA and proteins which were the major contaminants. RNA was removed by RNase treatment and proteins were removed by Proteinase-K treatment.

3.2.3 DNA Quantification by using Agarose gel electrophoresis

Concentration of DNA in the sample was determined by agarose gel electrophoresis with uncut lambda DNA on 0.8 per cent agarose gel and by comparison of the intensity of band staining with ethidium bromide.

3.2.4 DNA Amplification

The isolated and quantified DNA sample was subjected to DNA amplification by the use of following components.

3.2.4.1 Requirements

a) ISSR primers: A set of 15 ISSR primers composed wholly of defined, short tandem repeat sequences with anchor, and representing different microsatellites (di and tri-repeats) have been used as generic primers in PCR amplification of inter simple sequence repeat regions as per the method of Adawy *et al.*, (2004).

b) Template DNA: Purified DNA from Finger Millet leaf sample.

c) dNTPs: dNTPs mixture of 2.5 mM obtained from M/S Bangalore Genei Pvt. Ltd., Banglore.

d) Taq polymerase: 3.0 U/µl Taq DNA polymerase each obtained from M/S Bangalore Genei Pvt. Ltd., Banglore.

e) Taq buffer: Taq buffer 10X obtained from M/S Bangalore Genei Pvt. Ltd., Banglaore.

f) $MgCl_2$: $MgCl_2$ mixture of 25 mM each obtained from M/S Bangalore Genei Pvt. Ltd., Banglaore.

g) Thermal cycler: Eppendorf, Master cycler gradient supplied by Eppendorf gradient, 2231, Hamburg Germany was used for cyclic amplification of DNA.

Table 4: List of ISSR primers with their sequence.

Sr. No.	Primer	Primer sequence	GC Content	Tm^0 Value
		(5' – 3')	(%)	(^{0}C)
1.	UBC-807	AGA GAG AGA GAG AGA GT	47.1	45.0
2.	UBC-811	GAG AGA GAG AGA GAG AC	52.9	43.3
3.	UBC 812	GAG AGA GAG AGA GAG AC	52.9	44.4
4.	UBC-813	CTC TCT CTC TCT CTC TT	47.1	43.5
5.	UBC-814	CTC TCT CTC TCT CTC TA	47.1	41.4
6.	UBC-815	CTC TCT CTC TCT CTC TG	52.9	45.0
7.	UBC-816	CAC ACA CAC ACA CAC AT	47.1	51.2
8.	UBC-817	CAC ACA CAC ACA CAC AA	47.1	52.8
9.	UBC-818	CAC ACA CAC ACA CAC AG	52.9	52.1
10.	UBC-824	TCT CTC TCT CTC TCT CG	52.9	49.1
11.	UBC-825	ACA CAC ACA CAC ACA CT	47.1	49.3
12.	UBC-831	ATA TAT ATA TAT ATA TYA	5.55	20.2
13.	UBC-833	ATA TAT ATA TAT ATA TYG	11.1	55.0
14.	UBC-834	AGA GAG AGA GAG AGA GT	50.0	49.8

15.	UBC-841	GAG AGA GAG AGA GAG AC	55.5	45.7
16.	UBC-843	CTC TCT CTC TCT CTC TRA	50.0	37.6
17.	UBC-844	CTC TCT CTC TCT CTC TRC	55.5	39.4
18.	UBC-845	CTC TCT CTC TCT CTC TRG	55.5	43.4
19.	UBC-852	TCT CTC TCT CTC TCT CRA	50.0	44.9
20.	UBC-853	TCT CTC TCT CTC TCT CRT	50.0	54.0
21.	UBC-854	TCT CTC TCT CTC TCT CRG	55.5	51.1
22.	UBC-857	ACA CAC ACA CAC ACA CCG	55.5	57.1
23.	UBC-867	GGC GGC GGC GGC GGC GGC	100	58.6
24.	UBC-869	GTT GTT GTT GTT GTT GTT	33.3	51.0
25.	UBC-871	TAT TAT TAT TAT TAT TAT	00.0	32.2
26.	UBC-872	GAT AGA TAG ATA GAT A	25.0	28.9
27.	UBC-874	CCC TCC CTC CCT CCCT	75.0	33.0
28.	UBC-876	GAT AGA TAG ACA GAC A	37.5	36.4
29.	UBC-878	GGA TGG ATG GAT GGAT	50.0	29.0
30.	UBC-879	CTT CAC TTC ACT TCA	40.0	42.2
31.	UBC-881	GGG TGG GGT GGG GTG	66.6	66.5
32.	UBC-884	HBH AGA GAG AGA GAG AG	47.1	35.0
33.	UBC-885	HBH AGA GAG AGA GAG AG	52.9	41.7
34.	UBC-886	VDV CTC TCT CTC TCT CT	52.9	36.9
35.	UBC-889	DBD ACA CAC ACA CAC AC	47.1	39.4
36.	UBC-891	AGA TGT GTG TGT GTG TG	47.1	51.8
37.	UBC-893	NNN NNN NNN NNN NNN	-	42.0
38.	UBC-894	TGG TAG CTC TTG ATC ANN	-	62.4
39.	UBC-897	CCG ACT CGA GNN NNN NAT	-	56.2
40.	UBC-898	GAT CAA GCT TNN NNN NAT	-	47.3

B = (C, G, T) (i.e. not A) H = (A, C, T) (i.e. not G)

V = (A, C, G) (i.e. not T) D = (A, G, T) (i.e. not C)

Y = C OR T (i.e. not A,G) R = A OR G (i.e. not C,T)

Single letter abbreviations for mixed base positions

3.3.5.2 Stock solutions

a) ISSR primers

b) 50 ng μl^{-1} Template DNA

c) 3.0 U μl^{-1} Taq DNA polymerase.

3.3.5.3 Preparation of master mix

Initially the PCR master mix was standardized by changing the quantity of each component and the optimum concentration of each component in master mix which gave better amplification was chosen for amplification purpose. The master mix was distributed in 40 tubes (19 µl $tube^{-1}$) and 1 µl of template DNA of the respective genotype was added to make the total reaction volume to 20 µl and the mixture was given a short spin to mix the content.

Table 5: Master mixture for PCR

Components	Stock concentration	Vol. for one reaction/20 µl
Tris buffer	10X	2.0µl
$MgCl_2$	25 mM	0.5µl
dNTP mix	10 mM	1.0µl
Primer	25picomole	1.0µl
Taq DNA polymerase	3 U/µl	0.5µl
Template DNA	30-50 ng	1.0µl
Sterile Distilled water	-	14 µl
Total		20 µl

3.3.5.4 Thermal cycling

1. Sterile micro centrifuge tubes were numbered from 1 to 40.
2. 1µl of template DNA from individual genotypes was added to each tube.

3. 19 µl of master mix was added to all the tubes and was given short spin to mix the contents.
4. The tubes were placed in the thermal cycler for 30 cycles of PCR. Samples were held at 4^0C, in the thermal cycler, until the contents were loaded on to the gel for electrophoresis.

Table 6: Thermo Profile

	Temperature	**Period**	
Initial Denaturation	94°C	5 min.	
Denaturation	94°C	30 sec.	35 cycle
Annealing	40°C - 60°C	1 min.	
Extension	72°C	1 min.	
Final extension	72°C	7 min.	
Hold	4°C	-	

3.3.5.5 Separation of amplified product by agarose gel electrophoresis

The various chemicals and instruments used for agarose gel electrophoresis of amplified DNA samples are mentioned as under.

3.3.5.5.1 Requirement

a) Electrophoresis unit (Gel casting tray, gel comb, power pack)

b) Gel documentation system

c) Agarose

d) Tracking dye (Bromophenol blue)

e) Ethidium Bromide (1 µg ml^{-1})

f) 50 X TAE buffer.

3.3.5.5.2 Procedure

The amplified products in ISSR reaction were separated by electrophoresis in 1.5 per cent agarose gel (SRL, India), containing Ethidium Bromide in 1X TAE Buffer (pH 8.0) and separation were carried out by applying constant voltage of 100 volts for 1 hour. The standard DNA ladder used was Φ x174/Hae III digest. PCR and gel electrophoresis were carried out two times and only reproducible patterns were used for data analysis.

3.3.5.6 Photography and Gel documentation

The gels were photographed under UV light using Pentax K 312 nm camera. The images of gels were also taken by the documentation systems (Uvi-Tech. Fire reader, Cambridge, England) and saved in computer for further analysis.

3.3.6 Statistical analysis

ISSR markers across the 40 germplasm were scored for their presence (1) or absence (0) of bands for each primer. The binary data so generated was used to estimate the levels of polymorphism by dividing the number of polymorphic bands by the total number of scored bands. Jaccard's similarity co-efficients for each pairwise comparison between germplasm were calculated and similarity co-efficient matrix was generated. This matrix was subjected to Unweighted Pair Group Method for Arithmetic Average analysis (UPGMA) to construct a dendrogram. The similarity co-efficient analysis and dendrogram construction were carried out by using MVSP-A Multivariate Statistical Package-5785 (Version 3.1).

Distance matrix and dendrogram was constructed based on diversity coefficient generated from pooled data by using unweighted pair group method of arithmetic means (UPGMA), a

computer programme for distance estimation. Other parameters computed were,

$$\text{Per cent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \text{X } 100$$

CHAPTER IV

EXPERIMENTAL RESULTS

The experimental results obtained in the present investigation on genetic variability assessment in finger millet (*Eleusine corcana L.*) by using ISSR markers are presented in this chapter under the different headings.

4.1 Isolation of genomic DNA

The DNA was isolated from the 40 germplasms of finger millet which were numbered in order from N-1 to N-40. DNA was isolated by rapid DNA extraction method following the protocol of Doyle and Doyle (1990). The quality and quantity of DNA was ascertained through agarose gel electrophoresis. It was observed that good quality with sufficient quantity of DNA isolation was possible through rapid DNA extraction method (Plate-I).

4.2 Standardization of annealing temperature

The standard ISSR markers purchased with concentration of 5 pmoles/µl and were employed for optimization of annealing temperature. According to Tm value of particular ISSR primer the range of temperatures were adjusted in eppendorf PCR machine by following gradient PCR and the temperature at which the best results are obtained was used for thermal cycling. The standardized annealing temperature of 15 ISSR markers ranged from 40.4^{0}C to 56.7^{0}C. The optimized annealing temperature for each primer is given in Table 7.

Table 7: Optimization of annealing temperature of ISSR Markers

Sr. No.	Primer	Primer sequence (5' – 3')	Range of temp. (^{0}C)	Standardized Annealing temperature (^{0}C)
1.	UBC-807	AGA GAG AGA GAG AGA GT	45-55	50.4
2.	UBC-815	CTC TCT CTC TCT CTC TG	40-50	49.5
3.	UBC-816	CAC ACA CAC ACA CAC AT	45-55	51.4
4.	UBC-818	CAC ACA CAC ACA CAC AG	45-55	45.9
5.	UBC-824	TCT CTC TCT CTC TCT CG	45-55	56.7
6.	UBC-834	AGA GAG AGA GAG AGA GT	45-55	50.4
7.	UBC-841	GAG AGA GAG AGA GAG AC	42-52	47.4
8.	UBC-853	TCT CTC TCT CTC TCT CRT	45-55	54.4
9.	UBC-854	TCT CTC TCT CTC TCT CRG	45-55	54.8
10.	UBC-857	ACA CAC ACA CAC ACA CYG	45-55	51.7
11.	UBC-872	GAT AGA TAG ATA GAT A	40-50	43.0
12.	UBC-884	HBH AGA GAG AGA GAG AG	40-50	40.4
13.	UBC-885	BHB AGA GAG AGA GAG AG	40-50	40.7
14.	UBC-886	VDV CTC TCT CTC TCT CT	45-55	51.4
15.	UBC-891	HVH TGT GTG TGT GTG TG	45-55	52.8

Single letter abbreviations for mixed base positions

B = (C, G, T) (i.e. not A) H = (A, C, T) (i.e. not G)

V = (A, C, G) (i.e. not T) D = (A, G, T) (i.e. not C)

Y = C OR T (i.e. not A,G) R = A OR G (i.e. not C,T)

4.3 ISSR Analysis :

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. The ISSR pattern of genomic DNA of 40 germplasm were analyzed with respect to the fragments, informativeness of the markers and polymorphism for the assessment of genetic diversity present among the germplasm. For the present study 40 ISSR primers were screened. Out of that 15 primers showed better amplification and that were used for molecular characterization and assessment genetic diversity in finger millet.

Table 8: Primer wise amplification and percent polymorphism of finger millet germplasm.

Sr. No.	Primer Name	No. of Polymorphic Bands	No. of Monomorphic Bands	Total No. of Bands	Polymorphism %
1	UBC - 807	161	0	161	100
2	UBC - 815	104	0	104	100
3	UBC - 816	113	0	113	100
4	UBC - 818	68	40	108	62.96
5	UBC - 824	73	40	113	64.60
6	UBC - 834	17	40	57	29.82
7	UBC - 841	173	40	213	81.22
8	UBC - 853	102	0	102	100

9	UBC - 854	40	0	40	100
10	UBC - 857	87	0	87	100
11	UBC - 872	40	0	40	100
12	UBC - 884	162	40	202	80.20
13	UBC - 885	140	40	180	77.77
14	UBC - 886	107	40	147	72.79
15	UBC - 891	165	40	205	80.49
Total		1552	320	1876	83.32
Average		103.46	21.33	124.8	

4.4 Per cent polymorphism

Per cent polymorphism is the ratio of number of polymorphic DNA bands to the total number of DNA bands produced. The primer wise amplification detail of the genomic DNA of 40 finger millet germplasm and per cent polymorphism across the 15 ISSR primers is presented in the Table 8. A total of 1876 scorable DNA fragments were produced and among them 1552 DNA fragments were found to be polymorphic in the finger millet germplasm (Table 8). The minimum number of polymorphic fragments produced by the primer UBC-834 (17) while the maximum number of polymorphic fragments were produced by the primer UBC- 841 (173). Average number of polymorphic bands observed per primer were 103.46. The average percentage polymorphism across the 15 primers among the germplasm found to be 83.32.

4.5 Range of Amplification

The size of amplification ranged from 200 to 1650.

4.6 Polymorphic information content

The data on polymorphic information content is presented in Table 9. The ISSR profile generated by each primer was analyzed using standard DNA marker (1353 bp) and compared with their respective banding pattern.

Table 9: Polymorphic information content values and size of loci revealed by ISSR primers in finger millet.

Sr.No.	Primer Name	Range of Amplification (bp)	PIC
1	UBC - 807	600-1600	0.78
2	UBC - 815	500-1100	0.73
3	UBC - 816	550-1150	0.74
4	UBC - 818	300-1150	0.65
5	UBC - 824	200-1250	0.71
6	UBC - 834	350-900	0.71
7	UBC - 841	200-1000	0.88
8	UBC - 853	700-1250	0.72
9	UBC - 854	500-600	0.40
10	UBC - 857	900-1500	0.76
11	UBC - 872	100-300	0.20
12	UBC - 884	400-1650	0.84
13	UBC - 885	300-1000	0.78
14	UBC - 886	700-1650	0.71
15	UBC - 891	300-1400	0.81

The maximum polymorphic information content produced by the primer UBC-841 (0.88) followed by the primers UBC-884 (0.84). The minimum polymorphic information content was

produced by the primer UBC-872 (0.20). Average polymorphic information content was 0.70 among the all 40 germplasm of finger millet. It indicates that ISSR markers have a great potential to execute the polymorphism among the finger millet germplasm.

4.7 Primer wise amplification

The ISSR profiles of 40 germplasm of finger millet were computed individually for each primer by using standard DNA ladder. In the present investigation DNA ladder Φ x174/Hae III digest having amplification ranged from 72bp – 1353bp used for the ISSR analysis of all primers.

Primer UBC-807 displayed a total of 161 amplified fragments. All of them were polymorphic and exhibited 100 per cent polymorphism. Polymorphic information content of primer UBC-807 was 0.78. The amplification product ranged between 600-1600 bp.

The per cent polymorphism of UBC-815 primer was 100 per cent with total number of 104 fragments. The amplification range of this primer was found within 500-1100 bp. Polymorphic information content of primer UBC-815 was 0.73.

The primer UBC-816 produced a total of 113 DNA fragments and showed 100 per cent polymorphism. The polymorphic information content was 0.74. The range of amplification of this primer was from 550-1150bp.

The Primer UBC-818 showed 62.96 per cent polymorphism displaying 68 polymorphic and 40 monomorphic DNA fragments. The amplification range in between 300-1150 bp. The PIC value observed by this by primer were 0.65.

The per cent polymorphism of UBC-824 primer was 64.6 per cent with 73 polymorphic and 40 monomorphic DNA fragments produced. The amplification range of this primer was 200-1250 bp and polymorphic information content was 0.71.

The primer UBC-834 showed 29.82 per cent polymorphism and produced a total of 17 polymorphic and 40 monomorphic DNA fragments. The amplification of this primer ranged from 350-900 bp. Polymorphic information content of primer UBC-834 was 0.71.

The primer UBC-841 showed 83.09 per cent polymorphism among the 40 germplasm. A total of 213 DNA fragments were produced out of them 177 were polymorphic and 40 monomorphic. The DNA fragments were produced in between the range 200-1000 bp. Polymorphic information content of primer UBC-841 was 0.88.

The primer UBC-853 produced a total of 102 DNA fragments and which showed 100 per cent polymorphism across the 40 germplasm and the polymorphic information content was 0.72. The range of amplification of this primer was from 700-1250bp.

The UBC-854 primer revealed 100 per cent polymorphism with 40 polymorphic DNA fragments. The polymorphic information content was 0.40. The amplification range of this primer was found to be 500-600 bp.

The primer UBC-857 produced a total of 87 DNA fragments and which showed 100 per cent polymorphism across the 40 germplasm and the polymorphic information content was 0.76. The range of amplification of this primer was from 900-1500bp.

The UBC-872 primer revealed 100 per cent polymorphism with minimum number of polymorphic DNA fragments that is 40. The amplification range of this primer was found to be 100-300bp.

The UBC-884 primer showed 80.2 per cent polymorphism displaying 162 polymorphic and 40 monomorphic DNA fragments. The amplification range was in between 400-1650 bp.

The UBC-885 primer revealed 77.77 the per cent polymorphism of with 140 polymorphic and 40 monomorphic DNA fragments out of total of 180 DNA fragments. The amplification range of this primer was found to be 300-1000 bp.

The UBC-886 primer showed 72.79 per cent polymorphism displaying 107 polymorphic and 40 monomorphic DNA fragments. The amplification range was in between 700-1650 bp.

The primer UBC-891 showed 80.49 per cent polymorphism with 165 polymorphic and 40 monomorphic DNA fragments. The size of the DNA fragments ranged in between 300-1400 bp.

4.8 Genetic relationship among cultivars

The genetic distance was computed considering all the genotypes from the pooled data and the dendrogram was constructed. The pair-wise Jaccards similarity co-efficients for the genetic similarities among the 40 germplasm are presented in Table 10. The similarity co-efficient ranged from 0.197 (between germplasm Nagali-55 and KMR-204) to 0.679 (between germplasm VR-762 and PR-202) indicated the distinctness of these germplasm.

4.9 Cluster analysis

In the present study, 40 germplasm were subjected to cluster analysis for assessing the molecular diversity based on UPGMA analysis. The clustering pattern and the dendrogram constructed using Jaccard's similarity coefficient across the 40 finger millet germplasm is presented in Table -10 and Figure-1.

Table 10: Clustering pattern of 40 germplasm of finger millet

Cluster		No. of Genotypes	Name of the Genotype
I	IA	13	PR-1044, PR-202, VR-762, VR-708, VL-324, VL-149, L-481, OEB-265, L-5, OEB-54, IGPSM-18, IGPSM-10, GPU-69.
	IB	20	PNV-5, KMR-204, KOPN-235, ACCR-33, GOA-712, GSIS-01, MR-06, GPU-67, GPU-66, GPU-48, GPU-65, GPU-28, GPU-45, Kolhapur, Vakavali-02, Dapoli-1, Nagali-2RJ, Dapoli Safed Nagali-69, Nagali-67.
II	IIA	1	Nagali-55.
	IIB	6	Nagali-61, Nagali-66, Nagali-62, Nagali-56, Nagali-52, Nagali-35.

The cluster analysis band on the similarity co-efficient clearly distinguished all the 40 germplasm into two groups. The first cluster further subdivided into two subclasses. The first sub class of the first cluster containing 13 germplasm i.e. PR-1044,PR-202, VR-762, VR-708, VL-324, VL-149, L-481, OEB-265, L-5, OEB-54, IGPSM-18, IGPSM-10 and GPU-69, while the

second sub class consists of 20 germplasm i.e. PNV-5, KMR-204, KOPN-235, ACCR-33, GOA-712, GSIS-01, MR-06, GPU-67, GPU-66, GPU-48, GPU-65, GPU-28, GPU-45, Kolhapur, Vakavali-02, Dapoli-1, Nagali-2RJ, Dapoli Safed Nagali-69, Nagali-67. The second cluster further subdivided into two subclasses. The first sub class of the second cluster containing 1 germplasm i.e Nagali-55, while the second sub class consists of 6 germplasm i.e Nagali-61, Nagali-66, Nagali-62, Nagali-56, Nagali-52 and Nagali-35. VR-762 and PR 202 grouped together with maximum similarity co-efficient (0.679) followed by germplasm Nagali-55 and KMR-204 with minimum similarity coefficient (0.197). A genotype Nagali-55 occupied unique position in the cluster-II which was found to be highly differ from rest of the finger millet germplasm.

Table 11: Genetic distances based on ISSR pooled over the 15 primers in 40 germplasm of finger millet.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Nagali-35	1.000																																							
Nagali-52	0.528	1.000																																						
Nagali-55	0.389	0.406	1.000																																					
Nagali-56	0.429	0.471	0.475	1.000																																				
Nagali-61	0.438	0.422	0.385	0.513	1.000																																			
Nagali-62	0.409	0.390	0.333	0.520	0.571	1.000																																		
Nagali-66	0.329	0.414	0.348	0.523	0.463	0.614	1.000																																	
Nagali-67	0.342	0.391	0.344	0.477	0.407	0.447	0.462	1.000																																
Nagali-69	0.349	0.377	0.263	0.413	0.442	0.429	0.364	0.522	1.000																															
Nagali-2RJ	0.300	0.362	0.333	0.382	0.302	0.350	0.329	0.500	0.587	1.000																														
Dapoli-1	0.390	0.425	0.306	0.425	0.419	0.388	0.355	0.471	0.549	0.607	1.000																													
Dapoli Safed	0.379	0.410	0.299	0.410	0.438	0.425	0.363	0.395	0.611	0.552	0.606	1.000																												
Vakavali-02	0.263	0.387	0.357	0.365	0.284	0.205	0.232	0.323	0.314	0.404	0.452	0.455	1.000																											
Kolhapur	0.325	0.433	0.344	0.391	0.295	0.264	0.338	0.415	0.417	0.452	0.493	0.413	0.577	1.000																										
GPU-28	0.368	0.424	0.355	0.403	0.349	0.367	0.348	0.406	0.389	0.467	0.508	0.465	0.569	0.552	1.000																									
GPU-45	0.373	0.405	0.362	0.465	0.386	0.372	0.392	0.389	0.486	0.420	0.500	0.562	0.525	0.587	0.607	1.000																								
GPU-48	0.315	0.365	0.333	0.284	0.284	0.250	0.214	0.367	0.314	0.379	0.406	0.391	0.532	0.519	0.600	0.429	1.000																							
GPU-65	0.390	0.444	0.343	0.351	0.356	0.388	0.355	0.408	0.467	0.441	0.543	0.562	0.452	0.449	0.661	0.565	0.552	1.000																						
GPU-66	0.292	0.317	0.281	0.297	0.263	0.244	0.262	0.339	0.309	0.351	0.426	0.388	0.468	0.411	0.481	0.426	0.533	0.500	1.000																					
GPU-67	0.377	0.412	0.323	0.247	0.253	0.250	0.267	0.296	0.325	0.343	0.389	0.395	0.464	0.438	0.429	0.493	0.367	0.429	0.436	1.000																				
GPU-69	0.405	0.469	0.273	0.288	0.302	0.301	0.348	0.324	0.351	0.313	0.380	0.368	0.290	0.385	0.397	0.400	0.356	0.400	0.375	0.385	1.000																			
IGPSM-10	0.346	0.375	0.271	0.320	0.286	0.314	0.380	0.357	0.364	0.368	0.411	0.380	0.328	0.357	0.453	0.355	0.349	0.451	0.323	0.357	0.603	1.000																		
IGPSM-18	0.375	0.333	0.268	0.333	0.326	0.357	0.356	0.391	0.432	0.403	0.444	0.410	0.344	0.391	0.446	0.351	0.344	0.425	0.317	0.371	0.516	0.678	1.000																	
OEB-54	0.346	0.338	0.290	0.320	0.330	0.345	0.342	0.377	0.346	0.329	0.355	0.397	0.349	0.319	0.388	0.355	0.308	0.392	0.323	0.418	0.388	0.531	0.623	1.000																
OEB-265	0.314	0.281	0.286	0.262	0.250	0.247	0.246	0.300	0.333	0.267	0.303	0.333	0.308	0.345	0.357	0.303	0.388	0.344	0.327	0.300	0.407	0.473	0.519	0.473	1.000															
L-5	0.367	0.289	0.222	0.256	0.275	0.302	0.293	0.362	0.387	0.296	0.308	0.367	0.273	0.306	0.333	0.308	0.292	0.308	0.286	0.324	0.484	0.516	0.607	0.540	0.455	1.000														
L-481	0.318	0.244	0.227	0.259	0.348	0.318	0.329	0.361	0.317	0.263	0.293	0.302	0.257	0.256	0.315	0.325	0.222	0.277	0.269	0.380	0.333	0.423	0.478	0.530	0.292	0.515	1.000													
VL-149	0.342	0.231	0.229	0.280	0.310	0.341	0.319	0.353	0.342	0.324	0.370	0.377	0.242	0.278	0.364	0.351	0.262	0.333	0.317	0.314	0.324	0.418	0.500	0.484	0.345	0.516	0.607	1.000												
VL-324	0.320	0.348	0.215	0.254	0.244	0.272	0.313	0.393	0.397	0.361	0.368	0.356	0.316	0.371	0.361	0.310	0.364	0.348	0.385	0.393	0.482	0.467	0.459	0.419	0.365	0.526	0.444	0.441	1.000											
VR-708	0.293	0.279	0.242	0.279	0.280	0.278	0.265	0.383	0.388	0.350	0.338	0.366	0.352	0.277	0.350	0.300	0.352	0.338	0.346	0.339	0.373	0.433	0.450	0.509	0.438	0.518	0.534	0.509	0.551	1.000										
VR-762	0.397	0.320	0.236	0.269	0.330	0.270	0.273	0.338	0.400	0.368	0.373	0.416	0.269	0.267	0.274	0.288	0.269	0.355	0.344	0.338	0.409	0.307	0.375	0.380	0.306	0.448	0.403	0.418	0.443	0.509	1.000									
PR-202	0.364	0.301	0.214	0.284	0.284	0.239	0.270	0.319	0.365	0.309	0.356	0.400	0.328	0.300	0.309	0.303	0.306	0.375	0.368	0.400	0.369	0.362	0.439	0.424	0.305	0.500	0.448	0.468	0.500	0.577	**0.679**	1.000								
PR-1044	0.342	0.278	0.242	0.278	0.250	0.247	0.230	0.313	0.361	0.344	0.371	0.417	0.322	0.333	0.323	0.371	0.300	0.333	0.316	0.354	0.365	0.319	0.373	0.300	0.370	0.429	0.343	0.467	0.421	0.491	0.492	0.611	1.000							
MR-06	0.382	0.301	0.269	0.301	0.314	0.239	0.237	0.400	0.443	0.459	0.435	0.479	0.373	0.358	0.348	0.375	0.373	0.356	0.345	0.338	0.328	0.324	0.418	0.424	0.328	0.368	0.366	0.379	0.377	0.491	0.516	0.525	0.500	1.000						
GSIS-01	0.354	0.347	0.243	0.347	0.322	0.291	0.280	0.409	0.471	0.492	0.443	0.486	0.361	0.368	0.358	0.347	0.383	0.384	0.356	0.292	0.319	0.280	0.347	0.315	0.254	0.284	0.286	0.274	0.365	0.333	0.455	0.373	0.369	0.586	1.000					
GOA-712	0.264	0.282	0.216	0.333	0.341	0.295	0.253	0.391	0.413	0.424	0.387	0.410	0.284	0.333	0.270	0.351	0.265	0.268	0.221	0.280	0.306	0.269	0.299	0.338	0.206	0.289	0.397	0.352	0.290	0.318	0.414	0.377	0.373	0.532	0.590	1.000				
PNV-5	0.352	0.323	0.226	0.246	0.238	0.220	0.250	0.262	0.278	0.311	0.364	0.333	0.500	0.344	0.404	0.324	0.358	0.385	0.380	0.390	0.250	0.269	0.303	0.288	0.283	0.273	0.313	0.281	0.364	0.377	0.371	0.421	0.368	0.421	0.482	0.284	1.000			
ACCR-33	0.365	0.400	0.286	0.338	0.329	0.250	0.268	0.381	0.406	0.518	0.439	0.485	0.510	0.359	0.441	0.397	0.453	0.397	0.396	0.359	0.371	0.343	0.358	0.364	0.304	0.309	0.310	0.299	0.404	0.418	0.406	0.387	0.407	0.564	0.660	0.492	0.510	1.000		
KOPN-235	0.297	0.365	0.267	0.284	0.253	0.205	0.288	0.323	0.296	0.356	0.324	0.391	0.440	0.302	0.356	0.343	0.385	0.364	0.408	0.414	0.356	0.349	0.344	0.371	0.308	0.313	0.313	0.302	0.415	0.431	0.371	0.446	0.393	0.446	0.482	0.365	0.565	0.674	1.000	
KMR-204	0.296	0.301	**0.197**	0.319	0.345	0.313	0.288	0.358	0.403	0.435	0.435	0.544	0.421	0.319	0.413	0.414	0.397	0.356	0.345	0.319	0.328	0.343	0.357	0.362	0.305	0.310	0.347	0.338	0.377	0.414	0.343	0.385	0.426	0.500	0.508	0.508	0.421	0.654	0.558	1.000
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40

4.10 Dendrogram:-

The Dendrogram constructed using Jaccard's similarity coefficient using UPGMA (Unweighed Pair Group Method using Arithmetic Averages) grouped all genotypes into two main clusters is presented in figure 1.

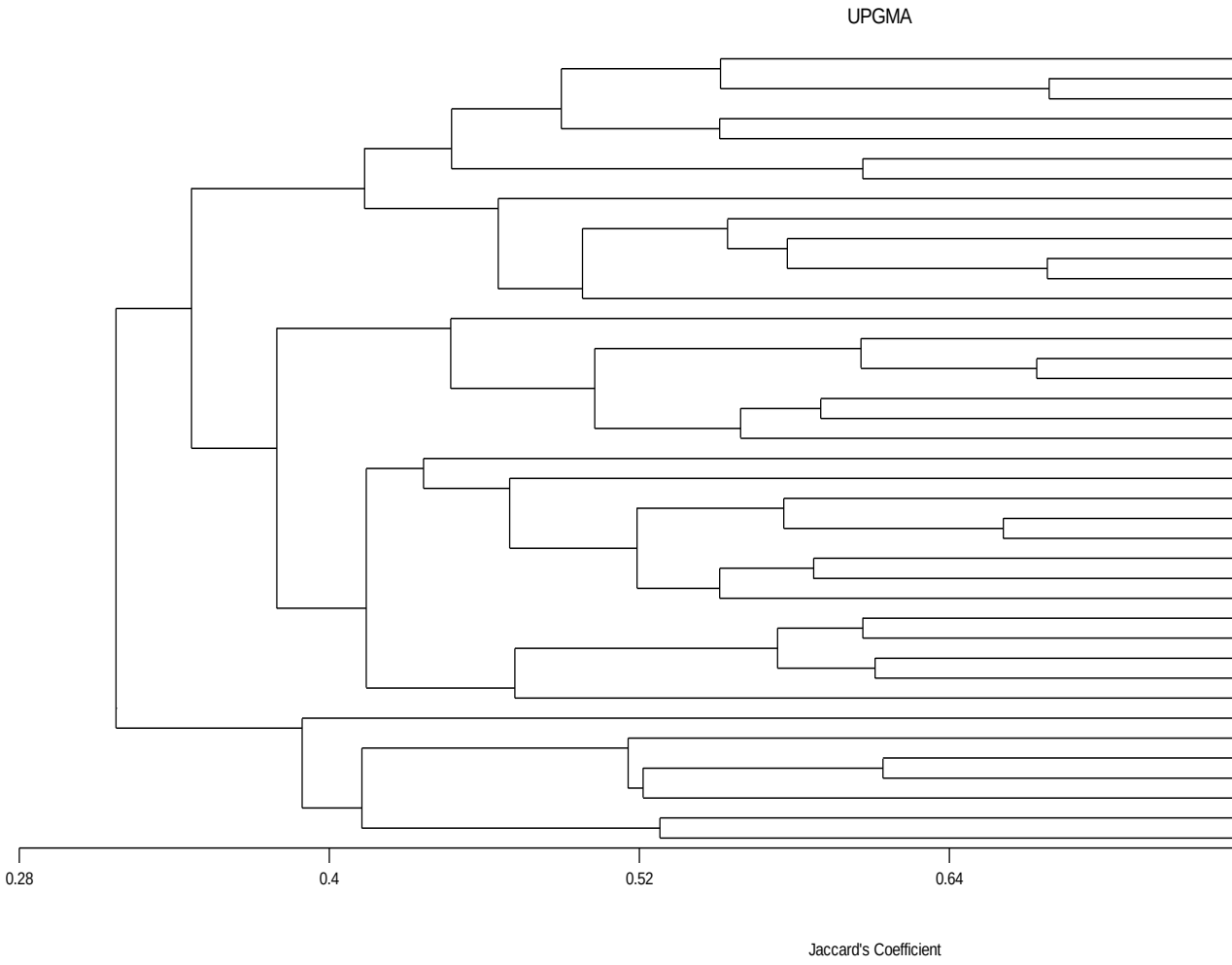

Fig. 2: Dendrogram constructed using Jaccards S

CHAPTER V

DISCUSSION

Present investigation was carried out with an objective to analyze the variability among the germplasm in finger millet (*Eleusine corcana* L.) through ISSR markers. Finger millet is an important cereal crop and there is need to improve this crop by means of modern tools like molecular techniques since traditional breeding methods have failed. Molecular techniques have great potential for use in quality control in breeding programme. The results obtained on polymorphic amplification products, genetic distance, discrimination power of the primers and grouping of 40 germplasm of finger millet are discussed in this chapter.

Genetic variability is normally assessed by common morphological traits. However, such traits are affected by effects of environment, development stage of the plant and the type of plant material it also requires several replications to establish the genotypic contributions. Hence, there is a need to find highly reliable and precise method for assessment of genetic variability with no environmental effects. Assessment of genetic variability 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 makes it possible to analyze the global organization of genetic variability within a species. Several statistical techniques are available for the analysis of genetic variability using DNA fingerprinting data. The present study, a set of 15 ISSR markers were used to assess the genetic variability of finger millet in 40 germplasm.

Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among the species. Many kinds of DNA based molecular markers such as RFLP, RAPD and AFLP etc. are available which detect polymorphism at the DNA level (Naga *et al.* 2012; Bezaweletaw., 2011; Gupta *et al.* 2010*;* Kumari *et al.* 2010; Sinha and Pande 2010; Panwar *et al.* 2010). The ISSR markers are multilocus, more reproducible, provide highly polymorphic fingerprints, are easy to develop in large numbers, have a simple assay, and are random, so that no prior sequence information is required. Due to these advantages they are effective for genetic variability analysis, fingerprinting and genome mapping. Hence, in the present study the ISSR markers were used to assess the genetic variability of finger millet.

The present study was undertaken to evaluate the pattern and the existence of genetic variability and relatedness among cultivars for genetic improvement of Ragi using ISSR markers. This would help in the identification and differentiation of various cultivars being grown for local consumption purpose. This will also contribute to maximize the selection of diverse parent cultivars and to broaden the germplasms base in the future finger millet breeding programmes. The information generated from this study will be used in identifying efficient strategies for the sustainable management of the genetic resources of finger millet.

During the course of investigation the DNA was isolated from the 40 germplasms of finger millet by rapid method following the protocol of Doyle and Doyle (1990). Similar DNA extraction method was used by Karad *et al.* (2013). Total DNA was extracted from the leaves of forty germplasms as per the

method described by Doyle and Doyle (1990) with some modifications. The quality and quantity of DNA was ascertained through agarose gel electrophoresis and it was observed that good quality with sufficient quantity of DNA was possible through rapid method. But the results were found similar to the results which were obtained by Gupta *et al.* (2013) who evaluated the three protocols, CTAB method, Sodium Dodecyl Sulfate (SDS) method and Sarcosyl method without using liquid nitrogen. All these three protocols were evaluated effectively to isolate the DNA from each of the four genotypes of finger millet. The quality and quantity of the DNA extracted was compared through agarose gel electrophoresis. It was found that CTAB and SDS both the methods are giving results at par with isolation of DNA however the superiority of SDS over the CTAB was in the amplification of PCR products. The suitability of the isolated DNA for performing PCR was checked using ISSR markers. Better resolution in more number of bands was observed in agarose gel for ISSR markers with DNA isolated using SDS method.

PCR requires very appropriate and little quantity of DNA (5 pm). If concentration is above or below the optimum there will improper amplification of the DNA, hence the DNA was diluted into the 50 µl of TE buffer and used as genomic DNA for PCR amplification. PCR master mix was standardized for each PCR component and the optimum concentration of each component in master mix which gave better amplification was used for further work. The PCR thermal cycling was performed at different annealing temperatures ranging from 40.0^{0}C to 56.7^{0}C which is different for each primer and its produced clear amplification and was used for further study.

ISSR markers are of great significance in interpreting polymorphism. ISSR primers do not require DNA sequence information for designing primers and they reveal variation at several loci simultaneously, thus constituting a multi locus marker system. The repeatability of ISSR-PCR is better than RAPD-PCR because ISSR primers are longer and hence have higher annealing temperature. Unlike AFLPs and SSRs, ISSRs are easier to handle and cost-effective as they can be resolved on agarose gels (Yadav *et al.* 2007). In this study, we have assessed the utility of variable ISSR markers to unique fingerprint and characterize the variability present in 40 germplasms in finger millet.

The present study utilized 40 germplasms for ISSR analysis with 40 random primers and out of them 15 gave scorable DNA bands and each of the 15 random primers revealed polymorphism. Similar results were reported by Prabhu and Ganeshan, (2013) who carried out the investigation to study the genetic diversity using ten SSR and thirty ISSR primers in forty ragi genotypes. Out of the ten SSR primers six showed polymorphism and out of the thirty ISSR primers used for molecular analysis, twenty primers showed polymorphism among forty ragi genotypes.

The primers produced high degree of polymorphism with an average of 83.32 per cent. Average 125 bands per primer were amplified. Among the 15 generic primers 7 primers UBC-807, UBC-815, UBC-816, UBC-853, UBC-854, UBC-857 and UBC-872 revealed 100 per cent polymorphism. The percentage of polymorphism across the finger millet genotypes ranged from 29.82-100 per cent. Prabhu and Ganeshan, (2013) recorded similar results in analysis of genetic diversity of 40 ragi

genotypes with 20 ISSR primers percentage of polymorphism ranged from 33-85 per cent among all genotypes. The result of Kumari and Pande (2010) indicated that the per cent polymorphism ranged from 6.6–100 per cent in eleven finger millet genotypes.

The monomorphic bands were constant bands and cannot be used to study diversity while polymorphic bands reveal differences and can be used to examine and establish systematic relationship among the genotypes. The amount of genetic variability recognized in such studies depends on the primer used and the amount of diversity among the genotypes. In this study the diversity per cent of polymorphism may be because of the highly divergent variety examined. More appropriately, the chosen primers were able to recognize the genetic differences and high level of polymorphism among the 40 germplasms.

Varietal differentiation based on DNA markers is an important aspect for further investigations such as construction of genetic maps, gene-tagging and other manipulations. However, the individual generic primers are of the little value in any of these studies, including analysis of genetic variability. Comparison of more primers generally provides additional confirmatory evidence for genetic variation reported by Bezaweletaw *et al.* (2011). Large numbers of primers have to be used to get a reasonable assessment of the status of a given genotype in relation to others and group the genotypes based on the DNA markers.

The genetic distance was computed considering the 40 germplasms from the pooled data. The overall range of the similarity among 40 germplasms of finger millet was found to be very wide ranging from 0.197 to 0.679 which indicates there was

high variability among the finger millet cultivars under study. Based on the similarity matrix and clustering pattern, the germplasms VR-762 and PR-202 were found to have maximum similarity coefficient 0.679 followed by IGPSM-10 and IGPSM-18 (0.678) thereby it indicating distinctness of these germplasms. While, the lowest similarity coefficient (0.197) were observed in between the germplasms Nagali-55 and KMR-204 which was suggesting a large differentiation in the germplasm of finger millet. Similar observations were also recorded by Gupta *et al* (2010) while carried out the study on assessment of genetic relatedness among three varieties of finger millet with variable seed coat colour using RAPD and ISSR markers. In this study molecular markers revealed a maximum similarities between PRM-701 and PRM-801 in terms of morphological, physiological and biochemical characteristics. The ISSR primers have a high potential to reveal polymorphism and to determine intra and inter genomic diversity Paul and Panneerselvam *et al*. (2013).

The Polymorphism Information Content (PIC) value was calculated for the 15 ISSR primers (Table 9). In the present study the maximum PIC information produced by the primer UBC-841 (0.88) while the minimum PIC value was given by the primer UBC-872 (0.20) the average PIC value obtained for each primer was 0.70. The similar result was obtained by Prabhu and Ganeshan, (2013) that is the PIC value was highest for the primer ISSR 44 (0.761) followed by primer ISSR 99 (0.756) while, the lowest PIC value was recorded by the primer ISSR 58 (0.345). The mean PIC value for ten ISSR primers was 0.588 obtained in forty ragi genotypes. The higher PIC value indicated the in formativeness of the primers. Hence, the primers UBC-891,

UBC-810, and UBC-825 can be of use in future studies in the field of taxonomical and genetic resource management.

The dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA after analysis of banding patterns generated by all the accessions with 15 primers across the 40 germplasm of finger millet genotypes. The dendrogram and similarity coefficient values give an idea about the nature of the individual sample in the whole sample set.

The cluster analysis was carried out based on the ISSR profile. The results based on the ISSR profile broadly grouped the 40 finger millet germplasm into two main clusters (I and II). The first cluster (I) was formed by the two subclasses. The first sub class of the first cluster containing 13 germplasm while the second sub class consists of 20 germplasm. The second cluster(II) further subdivided in to two subclasses. The first sub class of the second cluster containing 1germplasm while the second sub class consists of 6 germplasm. Similar results have been found by Gupta *et al*. (2012) for finger millet accessions in which the cluster one contain single variety PRM-1 and cluster two Contain two varieties PRM-701 and PRM-801 respectively based on ISSR analysis. This study could be used to identify the diverse genotypes like Nagali-55 and their use in hybridization programme of ragi. The genetic diversity in this study might be useful in future strategies for development of desired genotypes.

CHAPTER VI

SUMMARY AND CONCLUSION

Finger millet [*Eleusine coracana* (L.)], also known as Ragi, is cultivated for human consumption in Sub-Saharan Africa and South Asia. DNA was extracted from a single representative plant in each accessions was used for molecular profiling by using 15 polymorphic ISSR markers. Genetic variability was determined by using Multi Variate Statistical Package (MVSP). The current investigation was aimed to check the variability among 40 germplasms of finger millet through ISSR markers.

- ISSR profiles for all 40 germplasms of finger millet (*Eleusine corcana* L.) were generated with 15 ISSR primers. All of them gave scorable DNA fragments and revealed polymorphism.
- All together the primers generated total 1876 DNA fragments in the size range of 200 bp to 1650 bp, of which 1556 were polymorphic.
- The percentage of polymorphism across the finger millet germplasms was UBC-807 (100%), UBC-815 (100%), UBC-816 (100%), UBC-818 (62.96%), UBC-824 (64.60%), UBC-834 (29.82%), UBC-841 (81.22%), UBC-853 (100%), UBC-854 (100%), UBC-857 (100%), UBC-872 (100%), UBC-884 (80.20%), UBC-885 (77.77%), UBC-886 (72.79%) and UBC-891 (80.49%) and the average polymorphism across the 15 primers was as high as 83.32 per cent.
- The simple matching coefficient which indicated highest similarity index (0.679) was observed in between the germplasms PR-202 and VR-762, while the lowest similarity

index (0.197) was observed between Nagali-55 and KMR-204.

- The overall range of the similarity among 40 germplasms were found to be very wide ranging from 0.197 to 0.679 which indicates high variability among the finger millet germplasms under study.
- The cluster analysis based on ISSR data divided the germplasms in two main groups each having two sub clusters.
- Dendrogram revealed that genotype PR-202 and VR-762 showed very minimum differences between them at genotypic level. Whereas Nagali-55 and KMR-204 were observed with maximum genotypic difference.

Conclusion

The study indicated that ISSR markers are suitable for the assessment of genetic variability among different germplasms of finger millet. The ISSR analysis revealed substantial polymorphism in finger millet. The results of the present study indicated the efficiency of ISSR markers in investigating genetic variability at molecular level, which is important for detecting distinctness of germplasms also for the identification of desirable germplasms and its utilization for further breeding programme. Such information may be useful for selecting the diverse parents and monitoring the genetic diversity periodically for improvement of finger millet.

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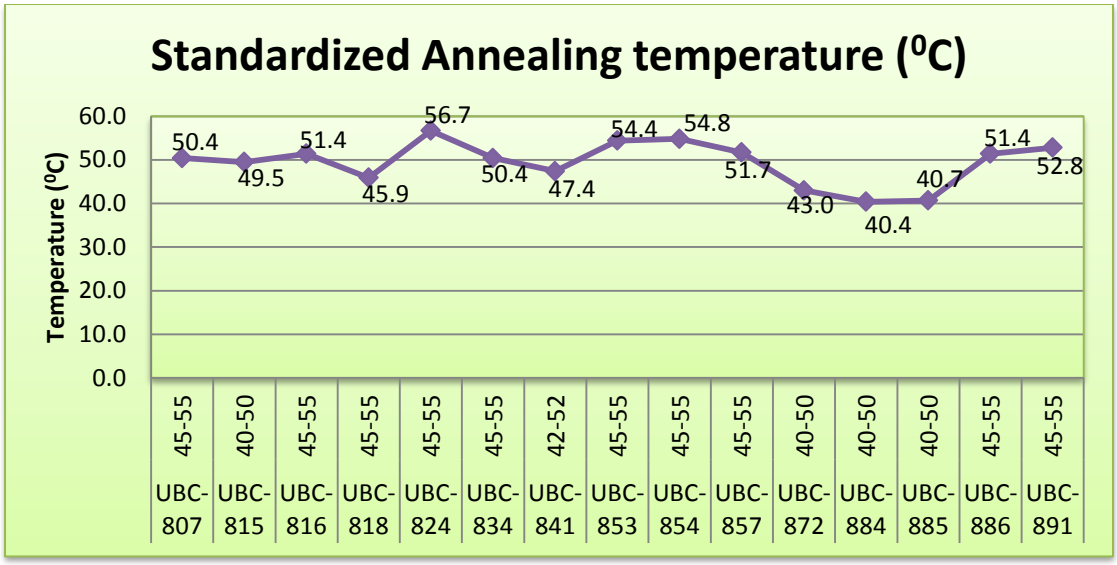

Fig. 1: Annealing temperatures (ºC) of ISSR marker.
(Maximum annealing temperature (56.7ºC) revealed by primer UBC-824, while minimum annealing temperature (40.4ºC) observed in primer UBC-884).

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Title of Thesis	:	Analysis of genetic variability among germplasm of finger millet by using ISSR marker.
Academic Year	:	2014-2016

ABSTRACT

The molecular marker technology has a great potential for assessing genetic variability and relationship among the selected germplasms. In the present study forty germplasm of finger millet showing distinct morphological differences were screened using 15 ISSR markers.

The DNA was extracted from the green leaf samples collected from 15 days old seedlings of finger millet from 40 gremplasms by rapid DNA extraction method. The combination of extraction buffer used was 200 mM Tris-HCl having pH 8.0, 25 mM EDTA, 250 mM NaCl which showed clear and specific banding pattern when subjected to PCR.

Optimum concentration of each component in master mix was used for further ISSR analysis. In which 10 mM (1µl) dNTPs concentration and Taq polymerase 3 U/µl (0.5 µl) gave better amplification. The annealing temperature ranging from 40.4^{0}C to 56.7^{0}C for 1 minute yielded good results.

The finger millet DNA showed better amplification with 15 ISSR primers studied. A total of 1876 bands were amplified and out of which 1552 were polymorphic which showed 83.32 % polymorphism. The primer UBC-834 showed 29.82 minimum per cent polymorphism while the average bands per primer were 124.8.

The ISSR profile generated by each of the primer was analyzed using standard DNA ladder (1353-310bp) and compared with their respective banding pattern. The average size of amplified fragment ranged from 200bp to 1650bp. The primer UBC-872 recorded minimum PIC value 0.20, whereas primer UBC-841 gave maximum PIC value 0.88 and average polymorphic information content is 0.70 among the all 40 germplasms. It indicates that

ISSR markers have a great potential to show the polymorphism among the finger millet germplasms.

The data of 40 germplasms of finger millet were used to generate pair-wise matrix based on the Jaccard's Similarity co-efficient. The genetic distance was calculated on the basis of pooled data and the dendrogram was constructed. The similarity co-efficient ranged from 0.197 (between germplasm Nagali-55 and KMR-204) to 0.679 (between germplasm VR-762 and PR-202) indicating the distinctness and similarities of these germplasm.

Cluster analysis was carried out based on UPGMA analysis and it divided 40 germplasms into two main clusters and each having two sub-clusters. The first sub-clusters of first major cluster comprised of 13 genotypes and the second sub-clusters also comprised of 20 genotypes. The first sub-clusters of second major cluster had 1 genotype. While the second sub-clusters of second major cluster consisted of 6 genotypes.

Introduction

Review of Literature

Material &Methods

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Summary & conclusion

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Abstract