Development of SSR (Simple Sequence Repeats) markers, physiological and molecular characterization to identify markers linked with drought tolerance traits in RILs (Recombinant Inbred Lines) of Finger millet (*Eleusine coracana Geartn*.).

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DEPARTMENT OF CROP PHYSIOLOGY
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Dedicated to my Family
Teachers and Friends

DEPARTMENT OF CROP PHYSIOLOGY
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BANGALORE

CERTIFICATE

This is to certify that the thesis entitled **"Development of SSR (Simple Sequence Repeats) markers, physiological and molecular characterization to identify markers linked with drought tolerance traits in RILs (Recombinant Inbred Lines) of Finger millet (*Eleusine coracana Geartn*.)."** submitted in partial fulfillment of **Doctor of Philosophy** in **Crop Physiology** to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by **MOHAN KUMAR, S.** under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bangalore
April 2010

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DEVELOPMENT OF SSR (SIMPLE SEQUENCE REPEATS) MARKERS, PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION TO IDENTIFY MARKERS LINKED WITH DROUGHT TOLERANCE TRAITS IN RILS (RECOMBINANT INBRED LINES) OF FINGER MILLET *(ELEUSINE CORACANA GEARTN.)*.

MOHAN KUMAR S

THESIS ABSTRACT

An investigation on "Development of SSR (simple sequence repeats) markers, physiological and molecular characterization to identify markers linked with drought tolerance traits in RILs (recombinant inbred lines) of finger millet (*Eleusine coracana geartn.*)" was carried out in the field and laboratory of Department of Crop Physiology, GKVK, UAS, Bangalore. The field experiment was taken up during *Kharif* 2005 using 150 recombinant inbred lines and their parental lines (IE2912 and IE 2885) laid out in randomized complete block design with three replications for physiological characterization.

The results from the first field experiment revealed that most of the physiological traits such as biomass, total leaf area, plant height, tiller number, Δ^{13}C, SCMR, MRC, root traits such as root length, root volume, root weight and all the traits varied significantly among the RILs except Δ^{13}C, SCMR and MRC. Further the traits normally distributed around the mean indicating their quantitative nature. To study the stability of these physiological traits across the season, second field experiment was taken up in similar way as that of first experiment. Most of the physiological traits varied significantly in the second experiment indicating their genotypic consistency over the seasons.

Results from the laboratory experiment which involved development/isolation of genomic SSR markers from finger millet revealed that out of 2200 colonies screened, 1021 recombinants were sequenced and annotated. Of these 645 sequences contained the microsatellite repeats, which varied in repeat type and size. The library consisted more of di-nucleotide repeats (67%) wherein CT-based repeat motifs were the most abundant (30%) followed by AG repeats (21%). A total of 323 primer pairs were designed in the flanking regions of microsatellite repeats, of which 156 primers showed locus specific amplification.

In addition to genomic SSRs, genic SSRs were also developed from dehydration stress specific EST library. From the annotation of 796 dehydration stress specific ESTs, 149 genic SSR primers were developed, of which 87 primers showed locus specificity. From the public domain, 228 rice and 31 finger millet SSRs validated in the finger millet which resulted locus specific amplification of 69 rice and 31 finger millet SSRs. Locus specific markers were further analysed for to parental polymorphism. The polymorphic markers were genotyped in mapping population. In total 15 polymorphic SSR and 95 RAPD marker loci were used to construct skeletal linkage map of finger millet with 14 linkage groups covering 6519.4 cM.

A total of 6, 8 and 11 markers were associated with SLA, leaf area and leaf weight respectively. Among the shoot traits, tiller number associated with maximum of 14 markers followed by plant height with 7 markers and stem weight 2 markers. Root traits were found to be associated with more number of markers compared to both leaf and shoot traits. Root length, root volume and root weight were found to be associated with 10, 13 and 11 markers respectively. These markers further validated and can be used for successful marker assisted selection for different physiological traits.

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ABBREVIATIONS

AFLP	- Amplified Fragment Length Polymorphism
Ca	- Ambient CO_2 Concentration
CGR	- Crop Growth Rate
CID	- Carbon Isotope Discrimination
DAS	- Days After Sowing
GCV	- Genotypic Co-efficient of Variance
HI	- Harvest Index
IRGA	- Infrared Gas Analyzer
IRMS	- Isotopic Ratio Mass Spectrometer
ISSR	- Inter Simple Sequence Repeats
LA	- Leaf Area
LOD	- Likelyhood of Odds
NAR	- Net Assimilation Rate
PCV	- Phenotypic Co-efficient of Variance
QTL	- Quantitative Trait Loci
RAPD	- Random Amplified Polymorphic DNA
RFLP	- Random Fragment Length Polymorphism
RH	- Relative Humidity
SCARs	- Sequence Characterized Amplified Regions
SLA	- Specific Leaf Area
SSR	- Simple Sequence Repeats
STSs	- Sequence Tagged Sites
T	- Transpiration rate
BM	- Total Biomass
WUE	-Water Use Efficiency
‰	-Parts per thousand
Δ^{13} C	- Discrimination against ^{13}C

Introduction

I INTRODUCTION

Finger millet (*Eleusine coracana Geartn.*) commonly called Ragi, is a subsistence crop mainly grown on dry lands. The grain has high nutritional value and excellent storage qualities, which make it an important famine food. Nutritionally the grain is rich in fiber, calcium and iron content. It is a recommended diet for the diabetics. The break through in this crop during the recent years is mainly done by the African germplasm. The first ragi-green revolution came in from the development of the Indaf series of ragi varieties, followed by the GPU series developed by the University Agricultural Sciences, Bangalore have increased the yields significantly.

The major constraint affecting the yield of ragi is blast caused by *Magnaporthe grisea*. Most of the finger millet land-races, and a number of other genotypes are highly susceptible to blast. In particular, neck and head blast inhibit grain formation/cause shriveling of grains, and in extreme cases yield losses up to 90% can result. Many blast resistant varieties have been developed in finger millet. But QTL identification for blast in this crop is also relevant.

In addition to blast, finger millet is also affected by drought. In general, plants have developed several mechanisms to cope with water-limited situations. However, from the agronomic point of view, drought tolerance should be associated with superior growth rates. From this perspective, a high ratio of carbon gain per unit water lost through transpiration, namely high water use efficiency (WUE), is hypothesized to conform a fitness advantage under drought stress (Cohen, 1970; Dudley, 1996; McKay *et al*., 2001).

According to the well-adopted growth model of John Passioura the differences in total biomass are a product of total transpiration and water use efficiency (TDM=T x WUE). This model emphasizes that increasing biomass is possible only when selection is made for both WUE and total water use (Richards *et al.*, 2002). Water use efficiency (WUE), the ratio of the biomass produced over a specific crop growth period to the total water transpired during the same period perhaps are the most important physiological traits to be considered for crop improvement in drought situation.

A significant progress in determining genetic variability in WUE was made with the establishment of theory linking carbon isotope discrimination ($\Delta^{13}C$) and season long WUE (Farqhuar and Richards, 1984; Farquhar *et al*., 1989a). This technique has been successfully adopted to assess the genetic variability in WUE in several important plants like cowpea (Ashok *et al*., 1999; Ismail and Hall, 1993), groundnut (Wright *et al*., 1998), wheat (Richards *et al*., 2002), sunflower (Virgona and Farquhar, 1996) including perennial species like spruce (Livingston *et al*., 1999) and Cashew (Raju, 2001) and also in rice (Impa *et al*., 2005).

. Water harvesting from deeper layers of the soil associated with deep root systems and an efficient use of water for biomass production are by far the most relevant physiological traits that can confer increased drought tolerance and superior growth rates. Root system morphology is one of the important components of drought resistance (Passioura, 1982). Species or cultivars that are drought tolerant differ morphologically or physiologically; this includes maximization of water uptake by deep and denser root system, minimization of water loss by stomatal closure and reduced leaf area (Kramer and Boyer, 1995).

O'Toole and Bland (1987) reviewed the genotypic variations in root systems and reported that plant root system is an important trait that needs to be exploited to enhance the drought tolerance in crop plants.

A deep and thick root system with high ratio of deep root weight to shoot weight and high deep root length density are factors contributing to resistance to intermittent drought stress in upland rice (Fukai and Cooper, 1995).The combination of relevant physiological traits must be pyramided in order to achieve an overall improvement in drought tolerance as well as enhanced productivity. However, breeding for these physiological traits is complex as these traits have polygenic inheritance and are also difficult to quantify. Thus, molecular breeding strategy where tightly linked DNA based molecular markers are used to identify the desirable genotype is expected to significantly complement the breeding processes to introgress relevant traits.

Quantitative trait loci (QTL) mapping is a complementary approach useful for identifying the genes underlying naturally occurring variation in traits (multigenic) such as those conferring drought adaptation. QTL mapping allows one to statistically identify chromosomal regions containing genetic factors contributing to variation in a polygenic trait (Lynch and Walsh, 1998; Alonso-Blanco and Koornneef, 2000). Once the tightly linked markers have been identified, they can be used to develop marker assisted selection strategy for breeding application. Molecular markers allow breeders to track genetic loci controlling drought resistance without having to measure the phenotype, thus reducing the need for extensive field-testing over space and time.

To identify relevant QTL for different physiological traits in finger millet requires trait specific mapping population and marker systems. Molecular markers are pre-requisite for assessing the polymorphism which further helps in the development of genetic map. The co-dominant markers such as Simple Sequence Repeats (SSR) are essential for detecting useful traits in segregating population but finger millet is a less investigated crop in terms of molecular breeding study and also that markers specific to the crop have not yet been developed.

With this background, a study was taken up with the major objective to develop SSR markers in finger millet and identifying markers linked to drought resistance traits in finger millet Recombinant inbred lines (RILs).

Objectives

1. Development of SSR markers in finger millet.
2. Phenotyping for drought resistance traits in Recombinant Inbred Lines of Finger millet
3. Molecular characterization of Recombinant Inbred Lines of Finger millet by different markers

Review of Literature

II. REVIEW OF LITERATURE

Finger millet, *Eleusine coracana* (L.) Gaertn. subsp. *coracana*, is an important coarse cereal in India and East Africa. The crop is adapted to a wide range of environments, can withstand significant levels of salinity, relatively resistant to dry land crop, and has few serious diseases. Finger millet is grown mainly by subsistence farmers and serves as a food security crop because of its high-nutritional value and excellent storage qualities. Under irrigated conditions in field trials, yields up to 5–6 metric tonnes/ha have been obtained (National Research Council,1996). However, yields in farmers' fields, usually sown with unimproved varieties, are commonly between 1000 and 2,000 kg/ha. Molecular breeding efforts in finger millet have been very limited. Crossing has long been impeded by the highly self-pollinating nature of the crop and the small flower size that has made hand-emasculation difficult. The development in the 1960s of hot water emasculation, combined with contact pollination, opened the way for the development of new crossbred varieties. However, even today, all released varieties in Africa are germplasm selections. In India, the hybridization of Indian and African varieties has resulted in the production and release of high-yielding 'Indaf' types that have proven popular with farmers.

Finger millet was domesticated >5,000 years ago from its wild progenitor, *E. coracana* subsp. *africana*. The 'A' genome donor is believed to be *Eleusine indica*, a ubiquitous weed of tropical and subtropical regions, but there are conflicting reports on the 'B' genome donor. Both *Eleusine tristachya* and *Eleusine floccifolia* have been considered potential B genome donors to *E. coracana* based on rDNA restriction patterns (Hilu and Johnson 1992) and genomic in situ hybridization (GISH) (Bisht and Mukai 2001), respectively. The latter study demonstrated that *E. tristachya* DNA hybridized to the same subset of *E. coracana* chromosomes as did *E. indica*, suggesting that *E. tristachya* is an 'A' genome species. GISH patterns of *E. floccifolia* and *E. indica*, on the other hand, did not overlap. Subspecies *coracana* and *africana* hybridize readily, with more than 80% of the meiotic cells in the hybrid forming bivalents, suggesting that the two genomes are highly homologous (Hiremath and Salimath 1992). Genetic knowledge and resources are scarce in finger millet. Finger millet is a tetraploid with genome composition AABB and a basic chromosome number of 9 (2*n* = 4*x* = 36). Genetic research in finger millet has been limited to studying the mode of inheritance of a few qualitative traits reviewed by Rachie and Peters (1977) and biodiversity analyses. Isozyme and DNA marker analyses have indicated that cultivated finger millet has a narrow genetic base and most likely went through a bottleneck during domestication (Hilu and Johnson,1992; Werth et al.,1994; Muza et al. 1995; Salimath et al.,1995). As expected, variation in the wild subsp. *africana* was considerably higher (Hilu and Johnson 1992; Werth et al. 1994). Although the finger millet germplasm pool remains largely uncharacterized, small-scale analyses of the nutritional value of seeds of wild and cultivated *E. coracana* lines have shown a wide variation in protein, calcium, and iron content (Barbeau and Hilu,1993; Vadivoo et al., 1998). Phenotypic variation for blast resistance, early vigor and other yield-related characters has also been observed.

Drought tolerance traits and their relevance in crop improvement

Carbon Isotope Discrimination

Plants discriminate against the heavy isotope of carbon (^{13}C) during photosynthesis resulting in the depletion of the ^{13}C content in the biomass (O' Leary, 1981). This deviation of the carbon isotopic ratio ($^{13}C/^{12}C$) of biomass from that of air, called discrimination ($\Delta^{13}C$) is related to the ratio of the partial pressures of CO_2 inside the leaf to that in the ambient air (Pi/Pa) (O' Leary, 1981; Farquhar *et al*, 1982; Farquhar *et al*, 1989a; Hubick and Farquhar, 1989) as follows;

$$\Delta^{13}C = a + (b - a)\ Pi/\ Pa,$$

where, a and b are fractionation against ^{13}C ($\delta^{13}C$) during diffusion through stomata; carboxylation by RuBisCO, respectively. Since WUE is also related to the CO_2 partial pressures, at a given VPD, a strong inter-relationship between $\Delta^{13}C$ and WUE is expected (Hubick and Farquhar, 1989; O'Leary, 1993).

There are two naturally occurring stable isotopes of carbon, ^{12}C and ^{13}C. Most of the carbon is ^{12}C (98.9%), with 1.1% being ^{13}C. The isotopes are unevenly distributed among and within different compound and this isotopic distribution can reveal information about the physical, chemical and metabolic processes involved in carbon transformations.

The overall abundance of ^{13}C relative to ^{12}C in plant tissue is commonly less than in the carbon of atmospheric CO_2, indicating that carbon isotope discrimination occurs during the incorporation of CO_2 into plant biomass. Because the isotopes are stable and non-radioactive in nature, the information is inherent in the ratio of abundance of carbon isotopes, presented by convention as $^{13}C/^{12}C$, is invariant as long as carbon is not lost.

The $\Delta^{13}C$ in plant samples is generally determined using a sophisticated analytical instrument called Isotope Ratio Mass Spectrometer (IRMS), specially designed for high precision measurements of the ratio R, defined as:

$$R = {}^{13}CO_2/{}^{12}CO_2$$

The plant material is converted to CO_2 by combustion to determine the isotope composition. In general R is low in organic sample. The atmosphere has a relatively higher fractionation value of around –7.8 per mil (‰), which is in comparison with a standard PDB (Pee Dee Belemnite, from North Carolina, USA). R in this standard is 0.0124 and in many of the plant material, it is approximately 0.012, suggesting a very minor changes in the R value, and hence R in a sample can be compared with that of standard and expressed as ^{13}C in units per mil or parts per thousand (‰).

$$\delta^{13}C = R_{sample} - R_{standard}/R_{standard} * 1000$$

Since the organic sample has less R-value than the standard, $\delta^{13}C$ of organic material is more negative, i.e., less ^{13}C content hence more discrimination and vice versa (O'Leary, 1984).

Importance and biochemical basis of $\Delta^{13}C$ and the relationship of Δ with WUE have extensively been studied (Farquhar *et al.,* 1982; Hubick *et al.,* 1988; Condon *et al.,* 1990; Read *et al.,* 1991). The range of $\Delta^{13}C$ values across the crop types, having different photosynthetic pathways, is given below:

Range of δ^{13}C composition

1. Atmosphere (Air) = -6.4 to –7.0%o
2. C_3 plants = -22 to –44%o
3. C_4 plants = -9 to –19%o
4. CAM plants = -11%o(approximate)

Δ^{13}C and WUE – relationship

Plants discriminate against the heavy isotope of carbon (Δ^{13}C) during the process of photosynthesis. However the extent of discrimination depends on the Pi and hence ^{13}C content in the plant samples has been emerged as a potential technique to quantify Pi and WUE. Several reports confirm the close relationship between Pi and Δ^{13}C; therefore Δ^{13}C could be a time-integrated estimate of Pi.

Since Pi/Pa ratios predominantly determine the variations in WUE and Δ^{13}C, a strong relationship between Δ^{13}C and WUE can be expected and explained by the following equation (Farquhar *et al.,* 1989b).

$$\mathbf{WUE = \{(1 - \theta)(b - d - \Delta)\}/1.6V(b - a)}$$

Where, θ is the proportion of fixed CO_2 lost in respiration, V is the leaf-air vapor pressure gradient.

An inverse relationship between A/g_s and Δ^{13}C (Meinzer *et al.,* 1990; Richards and Tieszen, 1993) and a positive relationship between Pi/Pa and Δ^{13}C signify that Pi determines the variability in Δ^{13}C (Hubick *et al.,* 1988; Gutterrez and Meinzer, 1994).

Although WUE and Δ^{13}C are related through the ratio of Pi/Pa, as well as with A/g_s (Condon *et al.,* 1990), because of diurnal and seasonal fluctuations in `A' and g_s, these parameters will not give an integrated estimate of WUE over a period of time (Hall *et al.,* 1993, Udayakumar and Prasad, 1994). From this context, Δ^{13}C is a dependable parameter as it is a reflection of time integrated estimate of carbon gain per unit transpiration, especially in C_3 plants. $\Delta^{13}C$ in whole plant dry matter appears to be reliable indicator of plant WUE in pot grown sunflower and negative relationship was obtained between these two traits in structural carbon both in well watered and drought conditions (Johnson *et al.,* 1993). In wheat, as in other C_3 species, genetic variability in $\Delta^{13}C$ is reflected in variation in WUE at both the leaf and at the whole-plant level (Condon and Richards, 1993).

Such a relationship between $\Delta^{13}C$ and WUE in several crop species as depicted (Table1) was not altered even when plants were subjected to abiotic stresses. Maintenance of the relative ranking of genotypes in control and stress implies that for WUE and $\Delta^{13}C$, genotype and environment interaction is low and the broad sense heritability is high (Hubick *et al.,* 1988; and Wright *et al.,* 1993). Due to these distinct advantages, $\Delta^{13}C$ appears to be a very reliable parameter for the identification of variability in WUE. This led to the initiation of several breeding programs to improve WUE using carbon isotope discrimination technique (Hall *et al.,* 1993; White, 1993).

Root traits

Knowledge from physiological studies indicated that the ability of the root systems to provide for evapotranspirational demand from deeper soil moisture is considered as one of the major drought resistant traits. A number of physiological and morphological traits have been reported to improve the performance of crops affected by drought. Root system morphology is one of the important components of drought resistance (Passioura, 1982). Species or cultivars that are drought tolerant differ morphologically or physiologically; this includes maximization of water uptake by deep and denser root system, minimization of water loss by stomatal closure and reduced leaf area (Kramer and Boyer, 1995)

Pinheiro and Da Matta, (2005) demonstrated that water stress developed more slowly in the drought tolerant clone than drought sensitive clones. Morphological traits such as leaf area and root mass to leaf area ratio were not associated with drought tolerance, instead much deeper root system of tolerant clones enabled them to gain access to water present in deeper layer of soil.

Table 1 : Relationship between $\Delta^{13}C$ and WUE in crop species

Species	Relationship			Reference
	r	p <	n	
Cereals and millets				
Wheat	-0.75	0.01	12	Farquhar and Richards, 1984
Wheat				Condon *et al.,* 1990
well watered plant	-0.74	0.01	16	
Watered stressed plant	-0.75	0.01	16	
Rice	-0.83	0.00001	34	Boominathan, 2001
Rice	-0.47	0.01	39	Nadaradjan *et al*., 2005
Rice	-0.815	0.05	6	Impa *et al.,* 2005
Pulses				
Cowpea	-0.93	0.05	5	Ismail and Hall, 1992
Soybean				White *et al.,* 1996
Irrigated	-0.68	-	-	
Water stressed	-0.80	-	-	
Navy bean	-0.80	0.05	-	Wright,1996
Chickpea	-0.83	0.001	12	Gangadhara, 1995
Cowpea	-0.68	0.02	11	Bindumadhava, 2000
Oilseeds				
Peanut	-0.81	0.01	34	Wright, 1996
Peanut	-0.64	0.05	8	Roy Stephen, 1995
Peanut	-0.69	0.01	17	Shashidhar, 2002
Others				
Kentucky Bluegrass	-0.55	0.01	11	Ebdon *et al.,* 1998
Crested wheat grass	-0.87	0.05	14	Read *et al.,* 1991

Large genetic variation in root morphology has been reported in germplasm adapted to different agro ecological conditions (O'Toole and Bland, 1987). Robertson *et al.,* (1985) commented that the available techniques for evaluating root traits are tedious and time consuming.

Furthermore, it is apparent that any plant's ability to withstand and survive drought stress is governed by its ability to harness water from deeper soil profiles or by its ability to reduce water loss through reduced leaf area or growth duration. Transpiration has been shown to be strongly related to total biomass accumulation and hence increasing total transpiration through improved water uptake has relevance (Angus and Herwaarden, 2001).

Under water scarce condition, extraction of water from deeper soil profiles has been recognized as a very important trait that can sustain the water requirement for transpiration, thereby enhancing biomass production (Yadav *et al*., 1997).

However, in areas where there is sufficient water availability in water extraction would exhaust the soil water resources resulting in the end season stress, (Condon *et al*., 2002) the deeper profiles, deeper root system would significantly increase the total biomass as well as yield (Sinclair and Muchow, 2001).

A crop that is able to increase the amount of water transpired relative to another crop grown in the same water-limiting conditions accumulates greater plant biomass. Increased water transpiration can result from increased soil water extraction by a deeper and/or denser root system. (Perry Miller, 1999)

Chang *et al*. (1972) reported that upland and lowland Rice shared common traits such as greater root length of main axis, larger root diameter and a lower degree of branching. When conditions are favorable for relatively rapid transpiration rate the total water loss is often associated with the efficiency of absorption of water from deeper soil profile. If soil moisture is not limiting this depends largely on the extent and efficiency of root system.

Price *et al.,* (1997) opined that root growth is an important component of the adaptation of rice to drought prone environments. Drought is a major constraint to the productivity of rice in upland ecosystems and the rice root system plays an important role in the regular Root and Transpiration rate.

The increased yield potential of clones of wheat (1Rsand 1BL) may be due to the greater root biomass and the observed higher transpiration rate (Moreno sevilla *et al.*, 1995).

Model calculations suggested that the deeper and more efficient roots are primarily responsible for the total water loss within the root zone when the near-surface soil layer approaches their wilting point.

Transpiration rate per unit of leaf area of loblolly pine and red cedar were found to increase as a function of the ratio of root surface to leaf surface. When the ratio of root surface to leaf surface of loblolly pine and red oak seedlings was varied by removing various amounts of root and leaf surface, the water loss per unit of leaf surface was found to be significantly correlated to the ratio of root surface to leaf surface (Johnson, 1948).

In a relationship between transpiration to root biomass, the white pine tree with high root biomass lost more water compared to red pine with low root biomass. (Marshall, 1946).Variation in the ratio of root surface might be expected to affect the rate of transpiration, removal of part of the root system of pine seedlings reduced transpiration, transpiration indicating the dependence of transpiration on root biomass (Johnson parker, 1948). Marshall and Maki (1972) demonstrated that transpiration of pine seedlings might be related to size of the root system.

Although the rate of transpiration per unit leaf area was similar between fast- and slow growing species, the rate of water uptake per unit root weight was three times higher for the fast-growing ones. This is due to the much larger leaf area: root weight ratio for those species compared to the slow-growing ones (Porter and Remkes, 1990).

Although root characteristics have been shown to be important factors influencing drought avoidance (O'Toole and Bland, 1987), little is known about their genetic control and their contribution to yield under drought conditions. This is largely due to the difficulty in properly investigating roots in a large number of plants, particularly in field experiment.

It would be rewarding to include relatively easily measured root traits like root length, root density, root weight and root pulling force are desirable parameters to exploit in breeding programs. (O'Toole and Soemartono, 1981). Ray *et al.* (1996) opined that root penetrating ability is an important factor for rice drought resistance in areas with soils subjected to both compaction and periodic water deficits. Breeding for root penetration ability is inhibited by difficulties associated with measuring root traits. Thanh *et al.* (1999) after finding positive correlations between the root -traits concluded that, the selection based on any of the root traits especially the easily measurable one, may provide breeders an opportunity to develop drought resistant upland rice varieties.

The resistance required to pull plants from the soil was correlated with root weight and branching (O'Toole and Soemartono, 1981). Pulling force in maize has been related to improve lodging resistance (Arihara and Crosbie, 1982).

The relevance of root traits in imparting drought tolerance

Any increase in the crop yield is associated with increase in transpirational water losses, so advantage of increased root characteristics help to enhance the soil water recovery there by increased total biomass and yield (Serraj and Sinclair, 2002).

Under drought, roots can adapt to continue growth while at the same time sending signals to shoot that exhibit growth above ground (Sharp *et al*., 2004). O'Toole and Bland (1987) reviewed the genotypic variations in root systems and reported that plant root systems is an important trait that needs to be exploited to enhance the drought tolerance in crop plants. A deep root system may also improve yield in less arid climates where occasional long intervals between rains results in depletion of water in upper soil layer (Taylor, 1980).

It was observed that the plants stayed cool amazingly even after sixteen days of imposing a long duration water stress in the field. Maintenance of higher level of transpiration is possible only by elongation of root further to reach untapped water in deeper layer of the soil. (Shashidhar *et al.*, 2000).

Drought tolerant genotype in wheat has significantly more roots in the crown region with the nodal and seminal roots concentrated close to the soil surface. The susceptible genotypes had fewer roots in the crown regions with the roots concentrated away from the soil surface (Kinyua *et al.,* 2003).

Ekanayake *et al.,* (1985a) reported that root length, root thickness, number of thick roots and root volume were significantly correlated to the field recovery from drought. Further they also opined that resistance to or tolerance of water stress in crop plants is the combined result of many interacting morphological and physiological characters like roots. Chang *et al*., (1986) investigated genetic variability in root characters among cultivars and reported that deep thick root systems avoid drought better than those with shallow thin root systems. Jeena and Mani (1990) studied root characters and grain yield on some upland rice varieties and indicated that apart from high root length density and root weight, the duration of crop was important for selecting drought tolerant genotypes. A deep and thick root system with high ratio of deep root weight to shoot weight and high deep root length density are factors contributing to resistance to intermittent drought stress in upland rice (Fukai and Cooper, 1995).

DNA Markers and Mapping

Molecular markers and marker mapping are part of the intrusive new genetics that is thrusting its way into all areas of modern biology from genomics to breeding, from transgenics to developmental biology, from systematics to ecology and even perhaps especially into plant and crop physiology. To put physiology on the map, there is an absolute need to first find the gene. The physiologist may argue that, almost by definition a gene is identified by a change in its function which is true, but it is also a true that until we map the genes or have a clear sign posts to their location in a linkage group we cannot do much with them, which and where the molecular markers come in. Over years different molecular marker system has evolved and utilized in several application (Table 2).

Molecular Markers

Molecular markers reveal neutral sites of variation at the DNA sequence level. By ‘neutral’ means, unlike morphological markers, these variations do not show themselves in the phenotype, and each might be nothing more than a single nucleotide difference in a gene or a piece of repetitive DNA (Neil Jones *et al*., 1997)

The advent of molecular markers for use as probes for genomic DNA has revolutionized the genetic analysis of crop plants and provided not only geneticists, but also physiologists, agronomists and breeders with valuable new tools to identify traits of importance in improving resistance to abiotic and biotic stresses (Quarrie, 1996).

Randomly Amplified Polymorphic DNA (RAPD)

This method of DNA polymorphism analysis was developed independently by two different groups (Welsh and McClelland, 1990; Williams *et al.,* 1990). This procedure detects nucleotide sequence polymorphism in a DNA amplification-based assay only a single primer of arbitrary sequences using Polymerase Chain Reaction (PCR). In this reaction, a single species of primer binds to the genomic DNA at different sites on opposite strands of the DNA template. If these sites are within amplifiable distance a fragment is amplified. The presence of each amplification product identifies complete or partial nucleotide sequence homology, between the genomic DNA and the oligonucleotide primer at each end of the amplified product (Fig.1). On an average, each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphism between individuals. The major advantage of this assay over RFLP method is that there is no prior requirement for DNA sequence information of the genome. The protocol is also relatively quick and easy to perform and uses fluorescence in lieu of radioactivity (Williams *et al.,* 1992). Because the RAPD technique is an amplification-based assay only nano gram quantities of DNA required and automation is feasible.

Table 2 : DNA marker systems that have been developed over the years

Sl No	Acronym	Technique	Reference
1	AFLP	Amplified Fragment Length polymorphism	Vos *et al*., 1995
2	AP-PCR	Arbitrary primed PCR	Welsh and McClelland, 1990
3	AS-PCR	Allele Specific PCR	Sarkar *et al*., 1990
4	CAPS	Cleaved Amplified Polymorphic Sequence	Lyamichev *et al*., 1993
5	DAF	DNA Amplification Fingerprinting	Caetano-Anolles *et al*., 1991
6	ISSR	Inter Simple Sequence Repeats	Zietkiewiez *et al*., 1994
7	MP-PCR	Microsatellite Primed PCR	Meyer *et al*., 1993
8	RAMS	Randomly Amplified Microsatellite	Ender *et al*., 1996
9	RAPD	Random Amplified Polymorphic DNA	Williams *et al*., 1990
10	REMAP	Retrotransposon Microsatellite Amplified Polymorphism	Kalendar *et al*., 1999
11	RFLP	Restriction fragment Length Polymorphism	Botstein *et al*., 1980
12	SAP	Specific Amplicon Polymorphism	Williams *et al*., 1991
13	SCAR	Sequence Characterized Amplified Region	Williams *et al*., 1991
14	SRAP	Sequence Related Amplification Polymorphism	Li and Quiros, 2001
15	SNP	Single Nucleotide Polymorphism	Nikiforov *et al*., 1994
16	SSCP	Single Stranded Conformation Polymorphism	Orita *et al*., 1989
17	SSLP	Microsatellite Simple Sequence Length Polymorphism	Rongwen *et al*., 1995
18	SSR	Simple Sequence Repeat	Hearne *et al*., 1992
19	STMS	Sequence Tagged Microsatellite Sites	Beckmann and Soller, 1990
20	STS	Sequence Tagged Sites	Fakuoka *et al*., 1994

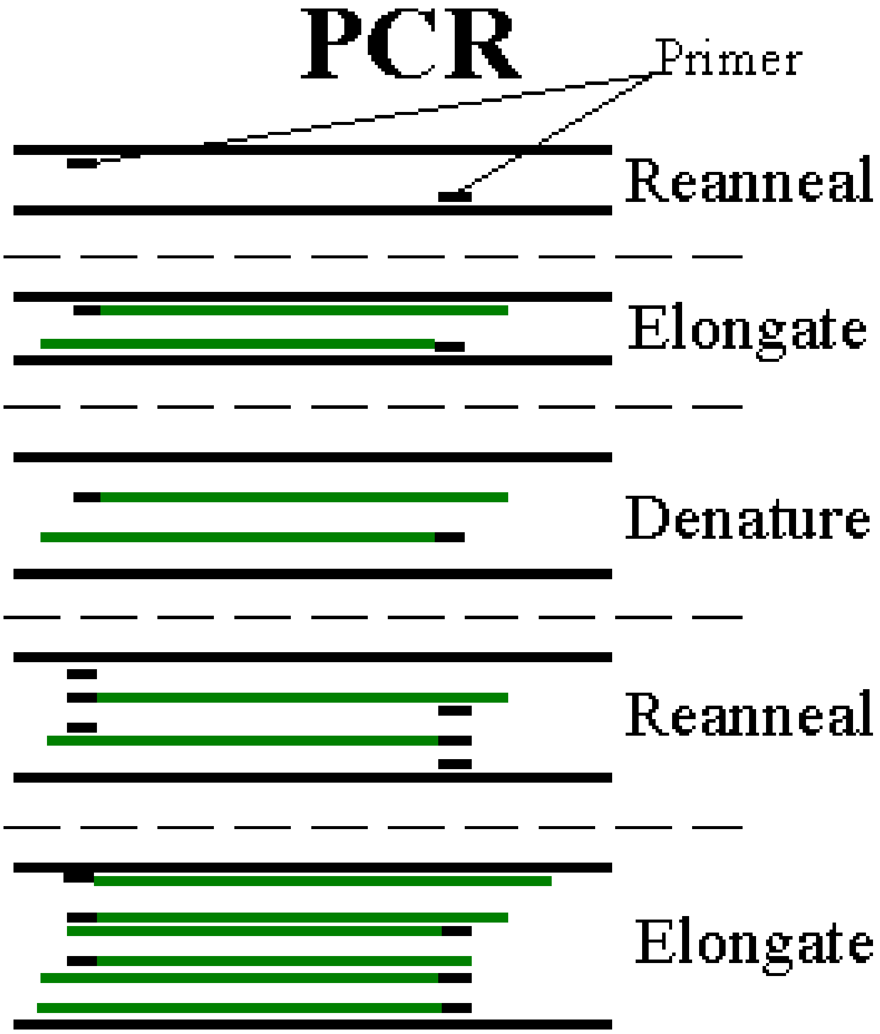


Fig. 1 : **Schematic representation of RAPD primer annealing to the locus in a genome**

This RAPD protocol is simple and fast. The procedure involves extraction of DNA from different accessions, by any of the standard protocol and amplifying the DNA by PCR using random primers. The profiles of accessions are scored for presence or absence of bands directly by running agarose gel, stained with ethidium bromide. Since RAPD approach is being adopted in the present study much of the literature reviewed pertains to use of this technique.

Fakuoka *et al.,* (1992) revealed that RAPDs are useful in detecting polymorphism in rice and superior to RFLPs for their technical simplicity in a study of classification of rice accessions into *japonica, indica* by 116 RAPD markers.

Yu and Nguyen, (1994) classified nine upland and four lowland rice cultivars using 42 RAPD primers and RAPD analysis was identical to the previous classification based on isozyme analysis. High level of polymorphism was found between *japonica* and *indica* subspecies.

A set of 18 accessions from an Indian scented rice (*Oryza sativa* L.) collection was subjected to random amplified polymorphic (RAPD) analysis. Polymerase Chain Reaction (PCR) with 10 arbitrary 10-mer oligonucleotide primers, applied to the 18 accessions, produced a total of 144 different marker bands of which 95.1 per cent were polymorphic. Visual examination of electrophoresis gels and analysis of banding patterns confirmed that many of the scented rice varieties under cultivation with similar name are genetically quite different. The RAPD analysis offered a rapid and reliable method for the estimation of variability between different accessions, which could be utilized by the breeder for further improvement of the scented rice genotypes. (Raghunathachari *et al*, 2000).

SSR markers

The genomes of higher organisms are with multiple copies of simple DNA in arrays of sequences, all arranged in arrays of vastly differing in size (Hancock, 1999). A good thumb rule is that bigger the repeat unit longer the array tends to be. These DNA sequences are of 3 types:

1. Major satellite DNAs: Long tracts upto several Mb, of well known families of repeat sequence elements.
2. Minisatellites: Blocks of repeated core elements longer than 10 to 15bp in tandem arrays up to 30Kb or more loger.
3. Microsatellites: The smallest class of simple sequence repeat elements in tracts less or much less than 1Kb.

The microsatellites are also called as short tandem repeats (STR) or simple sequence repeats (SSR) or simple sequence length polymorphisms. Typically they may be dinucleotides $(AC)_n$; trinucleotides $(TCT)_n$; tetranucleotide $(TATG)_n$ and so on, where n is the number of repeating units within the microsatellites locus. In addition to occurring at many different loci they can also be polyallelic. The $(AT)_n$ dinucleotides are the most abundant type of SSR in plants (Ma *et al.*, 1996). The methodology used to isolate an SSR at particular locus starts with the construction of the small insert genomic library. PCR amplification is used to generate DNA banding patterns on a gel and to reveal the polymorphism based on different number of repeats at the two alleles of a locus (Fig 2).

The marker thus has the advantage of being co-dominant. In addition they are simple, PCR based and extremely polymorphic, and highly informative due to number and frequency of alleles detected and to their ability to distinguish between closely related individuals. They find application as marker for mapping, cultivar identification, protecting germplasm, determination of hybridity, analysis of gene pool variation and as diagnostic markers for traits of economic value (Powell *et al.*, 1996).In rice molecular linkage map consisting of more than 500 microsatellite markers are now available for public (Temnykh *et al* ., 2001).

In plant systems, microsatellites or simple sequence repeats (SSRs) have become popular as DNA molecular markers. These sequences are abundant, ubiquitous, hyper variable, co-dominant and have been used for a variety of studies including genome mapping in several animal, insects and plant species (Gupta *et al.*, 1996; Gupta and Varshney, 2000).

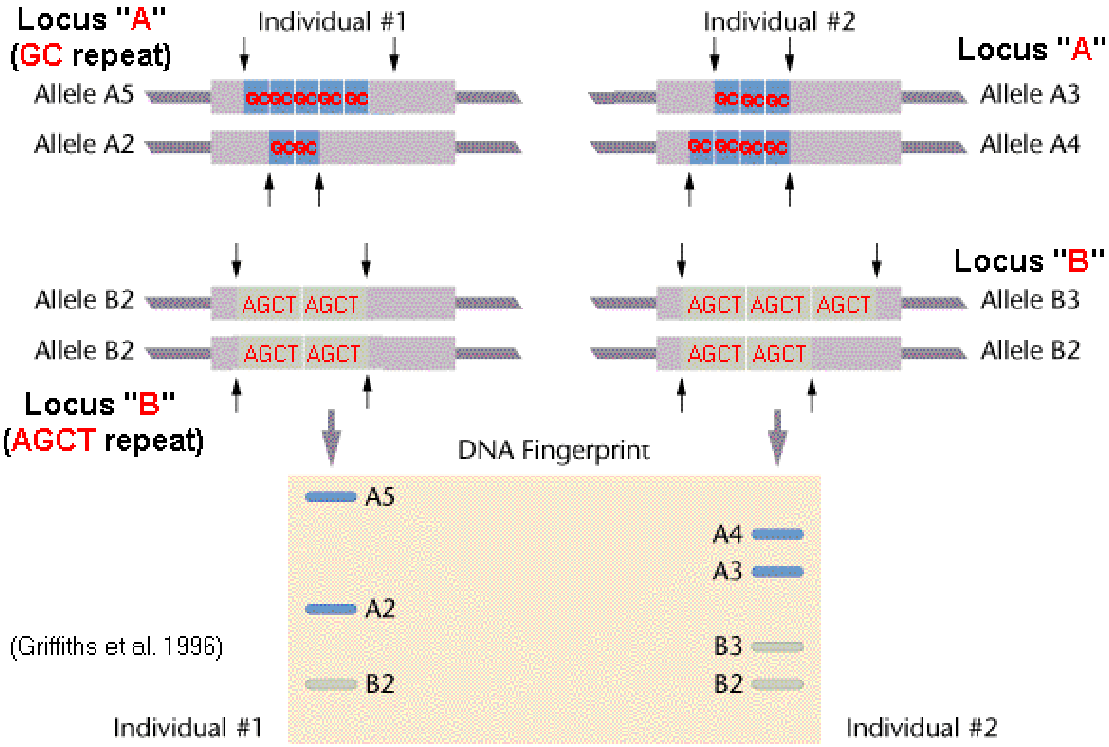


Fig. 2: Schematic representation of SSR protocol

Finger millet has great potential for improvement through the application of hybridization breeding. But one of the constraints in crop improvement through molecular breeding is the availability of codominant markers like Simple Sequence Repeats (SSRs) for which isolation of microsatellites/SSRs from the crop genome is crucial.

Isolation of Micro satellites/SSR markers from plant genomes

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 bases found in all prokaryotic and eukaryotic genomes analysed to date. They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism. The origin of such polymorphism is still under debate though it appears most likely to be due to slippage events during DNA replication (Schltterer & Tautz, 1992). Despite the fact that the mechanism of microsatellite evolution is still unclear, SSRs were being widely employed in many fields soon after their first description (Litt & Luty, 1989; Tautz 1989; Weber & May, 1989) because of the high variability which makes them very powerful genetic markers. Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms (Schuler *et al,* 1996; Knapik *et al*., 1998), but their applications span over different areas ranging from ancient and forensic DNA studies, to population genetics and conservation/management of biological resources (Jarne & Lagoda,1996).

Different methods for microsatellite isolation

Traditionally, microsatellite loci have been isolated from partial genomic libraries (selected for small insert size) of the species of interest, screening several thousands of clones through colony hybridization with repeat containing probes (Rassmann *et al*., 1991). Although relatively simple, especially for microsatellite rich genomes, this approach can turn out to be extremely tedious and inefficient for species with low microsatellite frequencies. Therefore, several alternative strategies have been devised in order to reduce the time invested in microsatellite isolation and to significantly increase yield (Table 3). To avoid library construction and screening, some researchers have proposed modifications of the randomly amplified polymorphic DNA (RAPD, Williams

Table 3 : **Library cost, time investment, and yield compared among the different protocols of micro satellite isolation**

Protocol	**Protocol set up* (US dollars)**	**Library* (US dollars)**	**Time**	**Yield**
Traditional	2000 -4000	< 400	1 month	Low
RAPD based	1000	< 100	1 week	Variable
Primer extension	1000-4000	< 400	2 weeks	Medium/High
Selective hybridization	1000-4000	< 400	1-2 weeks	Medium/High

et al. 1990) approach for the amplification of unknown microsatellites, by either using repeat-anchored random primers (Wu *et al*.,1994) or using RAPD primers and subsequent Southern hybridization of polymerase chain reaction (PCR) bands with microsatellite probes (Cifarelli *et al*.,1995; Richardson *et al*.,1995). Although not useful for single-locus analyses as no information on microsatellite flanking regions is obtained, these methods inspired alternative strategies for the identification of single microsatellite loci. Based on the observed abundance of repeat regions in RAPD amplicons, isolation of microsatellite regions is achieved simply by means of Southern hybridization of RAPD profiles with repeat containing probes, followed by the selective cloning of positive bands (Ender *et al*., 1996), or through cloning all the RAPD products and the screening of arrayed clones (Lunt *et al*.,1999). Other nonlibrary PCR-based strategies rely on the use of repeat-anchored primers to isolate and then sequence one (Fisher *et al*.,1996) or both regions (Lench *et al*.,1996; Cooper *et al*.,1997) flanking microsatellite repeats While all these methods provide, if successful, a quick alternative to laborious and time-consuming library screening, their use has not been that frequent.

A different strategy, based on primer extension, has been proposed for the production of libraries enriched in microsatellite loci for efficient enrichment of AC repeats yielding from 40-50 %(Ostrander *et al*.,1992) up to 100% positive clones (Paetkau,1999). These protocols involve a rather high number of steps which might explain their limited application. Many of these experiments concerned the isolation of dinucleotide repeat microsatellites, and it is unclear whether the primer-extension approach is effective also for tri- and tetranucleotide. The Ostrander protocol has not been tested for tri- or tetranucleotide repeat enrichment, whereas the Paetkau protocol produced 0-25% positive clones when using a tetranucleotide repeat primer in the extension step. The latter protocol has been reported, in the case of tetranucleotide enrichments, to produce multiple copies of the same clone, which might represent a problem when large numbers of micro satellites are needed. It is worth noting that both primer-extension protocols involve the production of a primary library in order to obtain a pool of single-strand circular DNA molecules for subsequent enrichment. In this step, for practical reasons, only a limited portion of the investigated genome is cloned, and so the population of inserts undergoes a severe bottleneck that results in loss of rare repeat motifs. With 60,000 clones in the primary library (Ostrander *et al.*,1992), in the case of a specific repeat motif with genomic frequency lower than 1%, only 600 loci (containing the desired repeat motif) will be represented in the enriched library. A significant amount of redundancy might therefore affect the above protocols. A further class of isolation methods is based on selective hybridization. The basic protocol was proposed by Karagyozov *et al.*,(1993), Armour *et al.*,(1994), Kijas *et al.*,(1994), and is relatively straightforward, although several modifications have been independently suggested by various authors in an attempt to further optimize crucial steps or to remove unnecessary procedures. In this strategy, enrichment efficiency ranged from 20% to 90%, in a large variety of taxa, from plants to vertebrates, using di-, tri-, and tetranucleotide probes. These protocols appear therefore to be efficient and widely applicable, and if working with microsatellite-rich organisms and dinucleotide probes, enrichment may even be so efficient as to allow microsatellite identification by directly sequencing random recombinant clones alone. Although relatively simple, all the selective hybridization methods can require some time in order to have the entire procedure up and running.

Selective hybridization

Selective hybridization is performed by using an oligonucleotide containing several tandem repeats of the motif to be enriched as a probe. The probe can be cross linked to a nylon membrane or can be biotinylated at the 5’ end, so that DNA hybridized with the probe can be selectively removed by using streptavidin-coated paramagnetic beads (Figure 3). The use of a biotinylated probe is generally preferable because in the liquid medium the probe is fully available for hybridization. In contrast, the nylon bound probe is partially cross-linked to the membrane, and therefore hybridizes less efficiently with the target DNA. Although different probe length, different hybridization and washing conditions are reported in the literature, the effect of these differences on microsatellite enrichment efficiency has not been extensively investigated. Nevertheless, Kandpal *et al.*, 1994 have standardized temperatures for stringency washes. After selective hybridization, captured fragments are recovered by PCR and cloned using standard methods. Finally recombinant clones are directly sequenced or Southern blotted

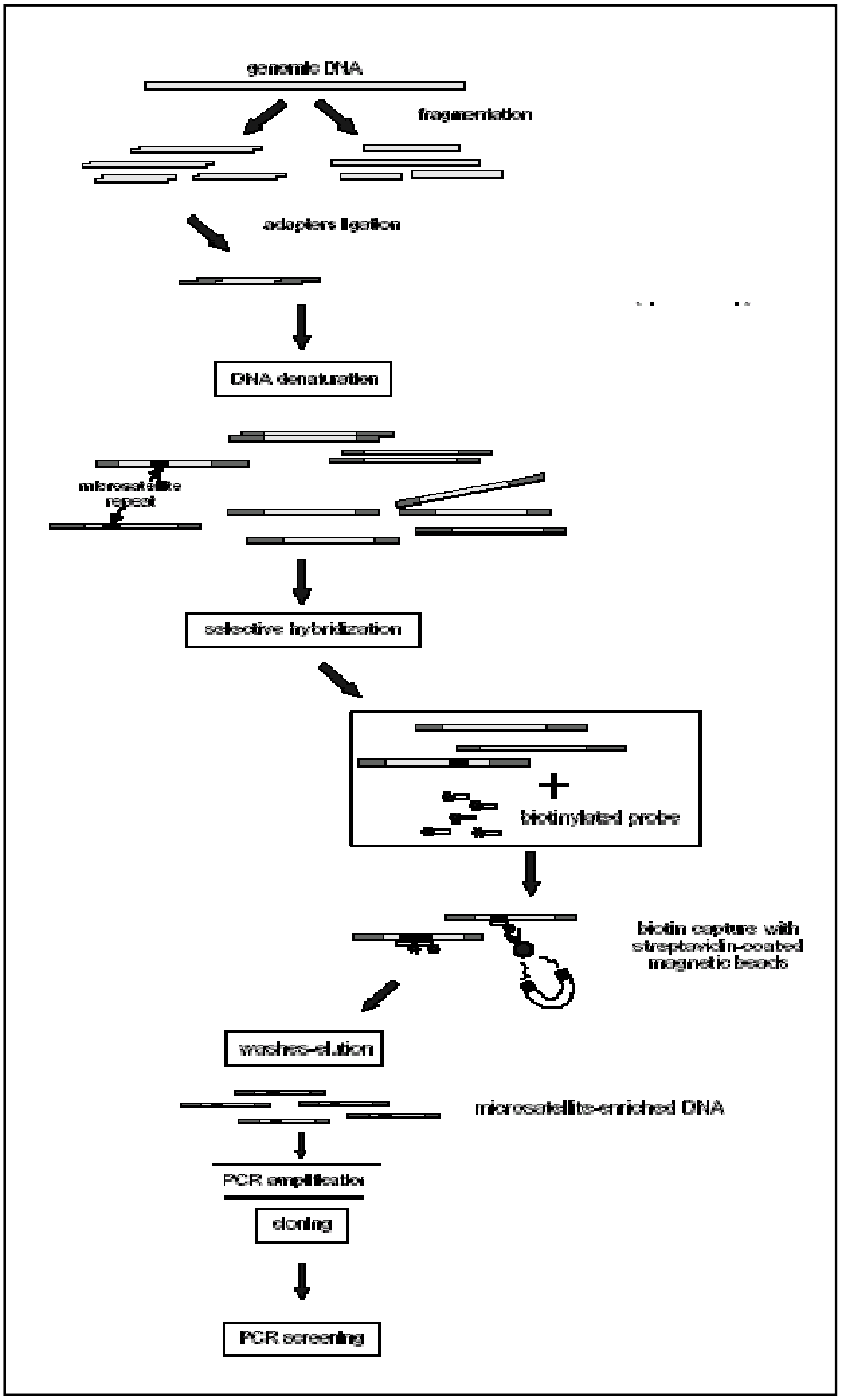


Fig. 3 : Schematic representation of selective hybridization protocol

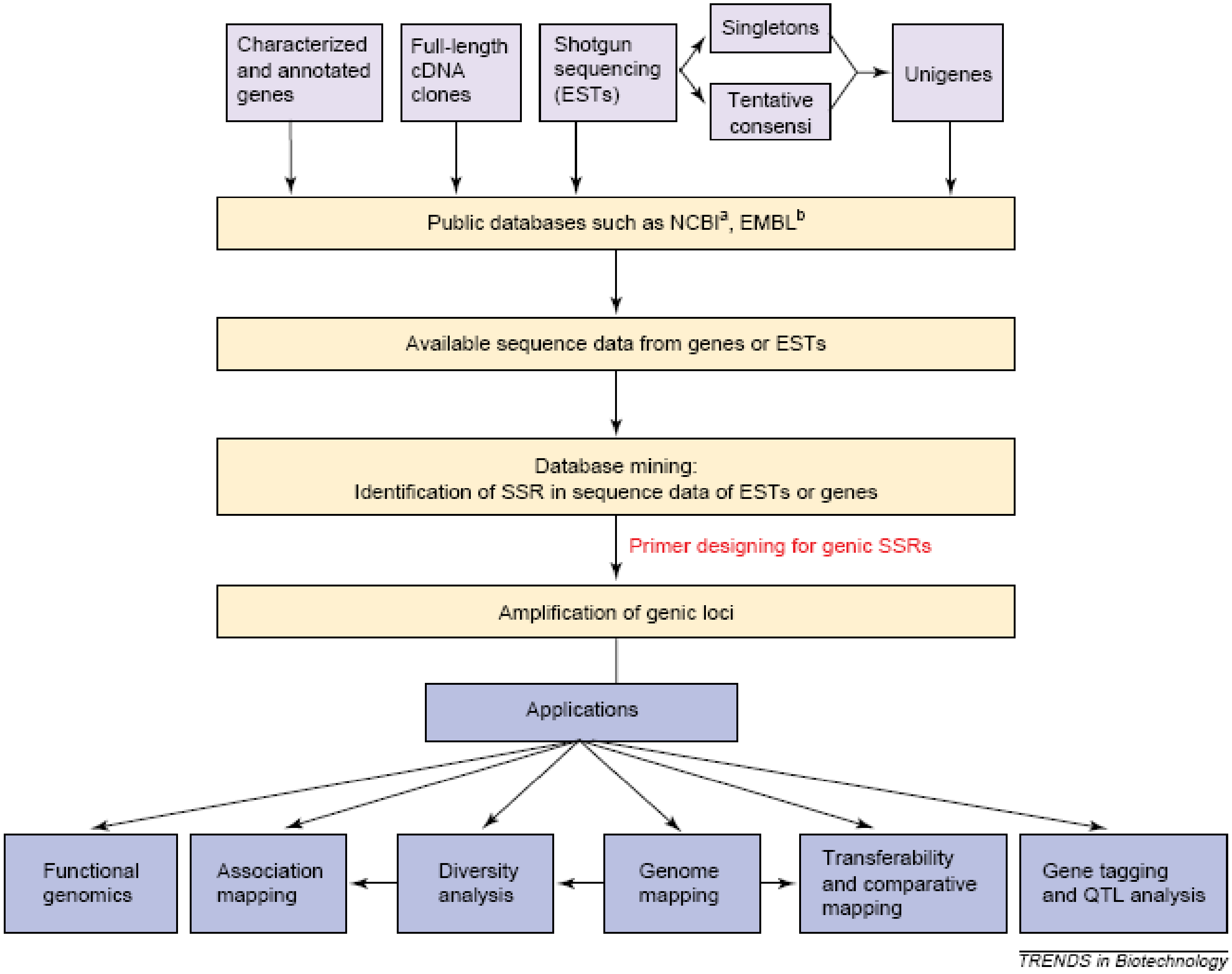
and probed. Alternatively, PCR screening of recombinants seems to be a good approach for mildly enriched libraries (Waldbieser, 1995). This approach involves two PCR reactions for every clone, using one primer for the vector and a second repeat-containing oligonucleotide.

Genic/EST SSRs

The establishment of expressed sequence tag (EST) sequencing projects for gene discovery programs in several plant species, a wealth of DNA sequence information has been generated and deposited in online databases (Rudd, 2003). In addition, sequence data for many fully characterized genes and full-length cDNA clones have been generated for some plant species such as rice (Kikuchi et al.,2003). By using some computer programs, the sequence data for ESTs, genes and cDNA clones can be downloaded from GenBank and scanned for identification of SSRs, which are typically referred to as EST-SSRs or genic micro satellites (Figure 4). Subsequently, locus-specific primers flanking EST- or genic SSRs can be designed to amplify the microsatellite loci present in the genes. Thus, the generation of (genic) SSR markers is relatively easy and inexpensive because they are a byproduct of the sequence data from genes or ESTs that are publicly available. However, the generation of genic SSR markers is largely limited to those species or close relatives for which there is a sufficiently large number of ESTs available. Genic SSRs have some intrinsic advantages over genomic SSRs because they are quickly obtained by electronic sorting, and are present in expressed regions of the genome. The usefulness of these genic SSRs also lies in their expected transferability because the primers are designed from the more conserved coding regions of the genome. Because of the advantages of genic SSR markers over genomic SSR markers and the public availability of large quantities of sequence data, genic SSRs have been identified, developed and used in a variety of studies for several plant species

Identification, frequency and distribution of genic SSRs

Identification of SSRs in gene sequences of plant species was carried out as early as 1993 by Morgante and Olivieri. However, at that time the volume of sequence data available for SSR analysis was limited and therefore only few genic SSRs were reported.



TRENDS in Biotechnology

Fig. 4 : A schematic representation of the development and application of genic simple sequence repeat (SSR) markers

Only one SSR per 64.6 kb in monocotyledonous and one per 21.2 kb in dicotyledonous species were identified (Wang et al., 1994). Subsequently, the sudden increase in the volume of sequence data generated from EST projects in several plant species facilitated the identification of genic SSRs in large numbers. For the identification of SSRs in publicly available EST and gene sequences, 'regular expression matching' or BLASTN tools were initially used in the FASTA or BLAST2 formatted sequences (Scott et al., 2000; Temnykh et al., 2000). Subsequently, several search modules or programs have been developed for recognition of SSR patterns in the sequence files (Table 4).

Because limited genomic sequence data are available for many plant species, EST databases have been screened for the development of genic SSRs. For example, ESTs have been scanned for the presence of SSRs in Arabidopsis (Morgante et al.,2002), cotton (Saha et al.,2003 and Han et al.,2004), Medicago species (Eujayl et al., 2004), soybean (Gao et al.,2003), sugarcane (Cordeiro et al. 2001), and cereals including barley (Thiel et al.,2003), maize (Varshney et al.,2002), rice (Temnykh et al.,2000;Varshney et al., 2002 and Kantety et al.,2002), sorghum,(Varshney et al., 2002 and Kantety et al.,2002) and wheat (Gupta et al., 2003 and Nicot et al., 2004). Varshney et al.,(2002) estimated the density of SSRs in expressed regions for 75.2 Mb of barley, 54.7 Mb of maize,43.9 Mb of rice, 3.7 Mb of rye, 41.6 Mb of sorghum and 37.5 Mb of wheat and found the overall average density of (redundant) SSRs to be 1 per 6.0 kb. Trinucleotide repeats (TNRs) are the most common, followed by either dinucleotide repeats (DNRs) or tetranucleotide repeats (TTNRs), depending on the report. For example, Varshney et al., reported that among cereal species, TNRs were the most frequent (54–78%) followed by DNRs (17.1–40.4%) and TTNRs (3–6%).

Level of polymorphism in the EST-SSR primers have been reported to be less compared to genomic SSRs in crop plants because of greater DNA sequence conservation in transcribed regions (Cho et al.,2000; Eujayl et al. ,2001 and Chabane et al.,2005) . It is noteworthy that for detection of polymorphism, EST-SSRs derived from 3' ESTs were found to be superior to those derived from 5'-ESTs (Scott et al., 2000 ; Gao et al., 2003 ; Holton et al., 2002 and Gao et al., 2003). During the process of cDNA generation (polyT priming), there is a preferential selection of untranslated regions (UTRs) within 3'-ESTs,

Table 4 : Tools for database mining

Script or program	**References**
MIcroSAtellite (MISA)	Thiel, T. et al. (2003)
SSRFinder	Gao, L.F. et al. (2003)
BuildSSR	Rungis, D. et al. (2004)
SSR Identification Tool (SSRIT)	Kantety, R.V. et al. (2002)
Tandem Repeat Finder (TRF)	Benson, G. (1999)
Tandem Repeat Occurrence Locator (TROLL)	Castelo, A.T. et al. (2002)
CUGIssr	http://www.genome.clemson.edu/projects/ssr/
Sputnik	http://abajian.net/sputnik/index.html
Modified Sputnik	Morgante, M. et al. (2002)
SSRSEARCH	ftp://ftp.gramene.org/pub/gramene/software/scripts/ssr.pl

resulting in more variation than in 5’-ESTs. Scott et al., (2000) also reported that there were polymorphism differences among microsatellites derived from the 3’ UTR (most polymorphic at cultivar level), 5’ UTR (most polymorphic between cultivar and species) and microsatellites within the coding sequence (most polymorphic between species and genera).

Comparative account on genic and genomic microsatellite markers

A comparative analysis of genomic SSRs and genic SSRs reveals advantages to both; however, because of lower polymorphism, EST-SSRs are not as efficient as genomic SSRs for distinguishing the closely related genotypes (Gupta and Varshney, 2000). Furthermore, the development of genic SSRs is restricted to those species for which there are sufficient sequence data (for ESTs or genes) available because SSRs are present in only 2% to 5% of the unigenes examined. Nevertheless, EST-SSR markers developed for a given species can successfully be used in a related species for a variety of purposes, including fingerprinting or diversity studies, comparative mapping and marker assisted selection. Genic-SSR and genomic SSR markers tend to be complementary for genome mapping, with genic microsatellites being less polymorphic but concentrated in the gene-rich regions. For assessment of functional diversity, the genic SSRs are useful; however, because of higher polymorphism, genomic SSRs are superior for fingerprinting or varietal identification studies.

QTL analysis

The process of QTL analysis mainly requires the following four factors:

1. A suitable mapping population of phenotypically contrasting parents
2. A linkage map of molecular markers
3. Mapping methods and software
4. Reliable phenotypic screening methods and generation of phenotypic data

Suitable mapping population

It would be always advantageous using populations of early generations such as F_2, F_3, Back cross population etc. since the development of these populations require only 2 to 3 years compared to the late generation populations such as F_6, F_8, RIL, BC inbred lines etc. which requires about 7 to 8 years (Fig.5). And development of these early generations are not a costly affair as in case of the doubled haploid lines. However predictions made involving early generations would be misleading because of the camouflaging effect of major genes on minor genes in early generations. Continues inbreeding to evolve Recombinant Inbreed Lines (RILs) can eliminate this camouflaging effect. Thus, RILs can remain as the best choice of population for QTL analysis. As an alternative, doubled haploid lines (DHLs) can also be used. The inherent homozygosity prevailing in the individuals of these two populations make the RILs & DHLs as immortals and help to have as many replications as required.

Linkage Map

Since the development of markers first allowed the construction of saturated linkages maps (Botstein *et al*., 1980), it has been clear that the technology of quantitative trait locus (QTL) analysis could be usefully employed to analyze the genetics of complex traits. Genetic map construction requires the following steps a) selection of the appropriate mapping population b) Calculating the pairwise recombination frequencies using these populations c) Establishment of linkage groups and estimation of map distances d) Determination of map order.

Large mapping populations are often characterized by different marker systems, so the map construction has become computerized. Computer packages such as Linkage 1 (Suiter *et al*., 1983), GMendel (Echt *et al*., 1992), Mapmaker (Lander and Botstein, 1986; Lander *et al*., 1987) and JoinMap (Stam, 1993) have been developed to aid in the analysis of genetic data for map construction (Staub and Serquen, 1996).

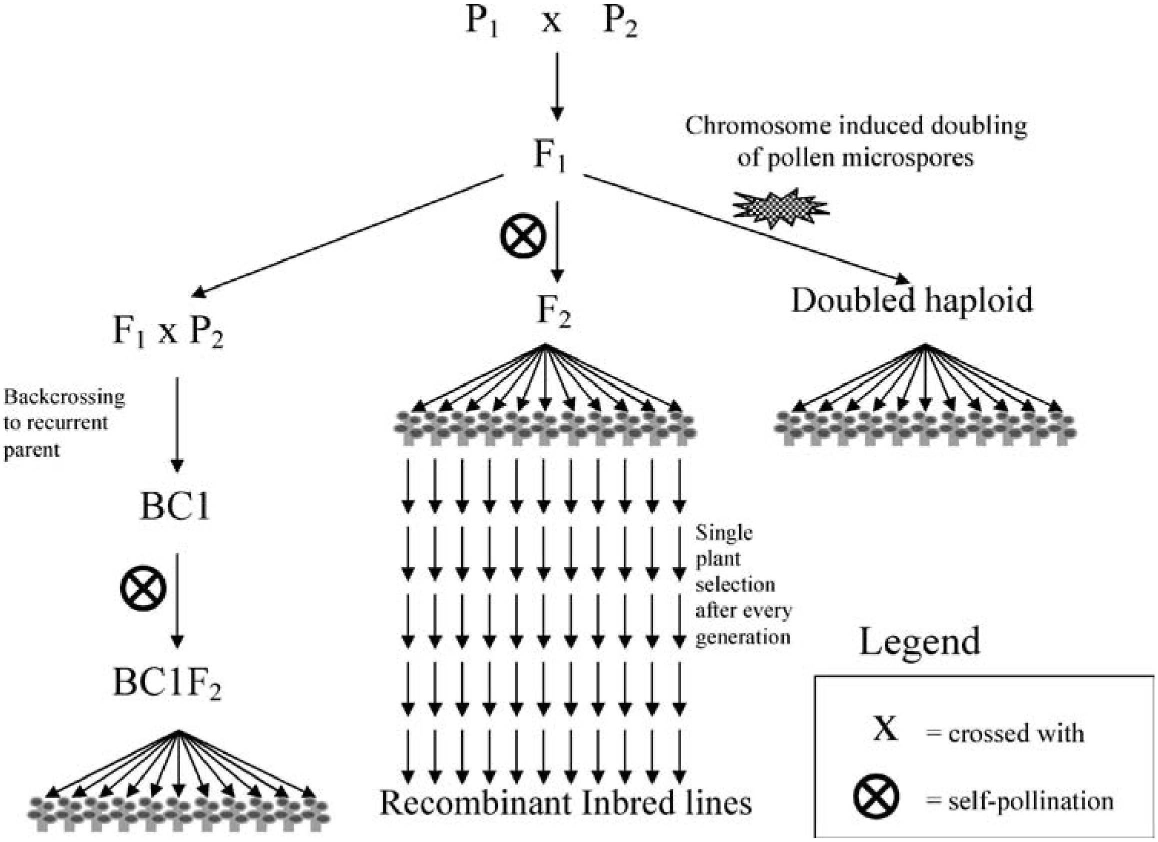


Fig. 5 : Flow chart for development of mapping populations

Mapping methods and software

The basis of all QTL detection, regard less of the crop to which it is applied is the identification of association between genetically determined phenotypes and specific genetic markers. The possible methods of analysis to detect QTL include 1) single Marker Analysis (Marker Trait Method) 2) Interval analysis

QTL mapping methods

QTL mapping methods accounts to 3-step recipe

- → Scan the entire genome with a dense collection of markers
- → Calculate an appropriate linkage statistic S(x) at each position x along the genome
- → Identify the regions in which the statistic 'S' shows a significant deviation from what would be expected under independent assortment.

The association between quantitative trait variation and marker segregation pattern can be carried out by the following methods:

1. Single Markers analysis (SMA)

SMA is the method used in earliest studies on QTL mapping (Edwards *et al.*, 1987; Weller *et al.*, 1988). In this method one marker is involved at a time to find the QTL – Marker association (Fig. 6). This single marker analysis can be implemented as a simple t test, ANNOVA, linear regression and likelihood ratio test and maximum likelihood estimation (Haley & Knott, 1992; Nienhuis *et al.*, 1987; Wang *et al.*, 1994). SMA can be performed using common statistical softwares.

2. Interval Mapping (IM)

IM is considered as a second level of QTL mapping. QTL mapping by this method requires a prior construction of a marker genetic map. The interval mapping approach is based on the joint frequencies of a pair of adjacent markers & a putative QTL flanked by the two markers (Fig. 7).

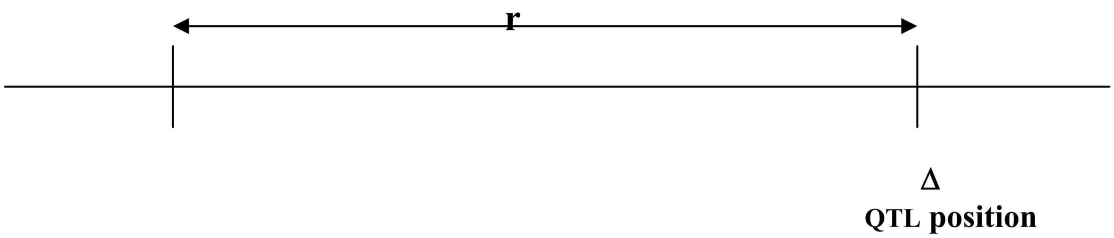


Fig 6 : Association of a marker with a putative QTL

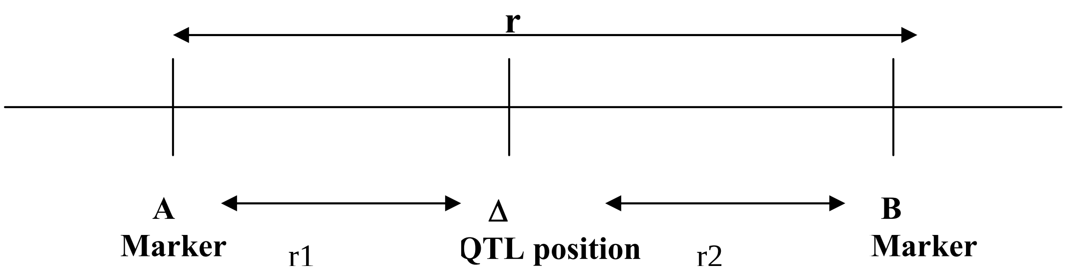


Fig. 7 : Association of a putative QTL to two flanking markers

Interval Mapping can be done by the following methods:

Likelihood approach - Lander & Botstien, 1989
Regression approach - Knapp *et al.*, 1990
Combination of likely hood & regression approach - Zeng, 1994

Multiple QTL Mapping:

- Composite Interval mapping
- Multiple Interval mapping

Both SMA & IM are biased when multiple QTLs are linked to the marker or interval being considered. To deal with multiple QTL problems, Jansen (1993), Rodolphe & Lefort, (1993) and Zeng, (1993) independently proposed the idea of combining simple interval mapping with multiple regression analysis in mapping.

Composite interval mapping (CIM)

This method evaluates the possibility of a target QTL at multiple analysis points across each intermarker interval. However at each point it also includes the effect of one or more background markers. The inclusion of a background marker in the analysis helps in one of two ways, depending on whether the background marker & the target interval are linked. If they are not linked, inclusion of a background marker makes the analysis more sensitive to the presence of a QTL in the target interval. If they are linked inclusion of background marker may help to separate target QTL from other linked QTL on the far side of the background marker (Zeng, 1993 & 1994).

Multiple Interval mapping (MIM)

This method uses multiple marker intervals simultaneously to fit multiple putative QTL directly in the model for mapping QTL. With MIM approach the precision and the power of QTL mapping could be improved. Also, epistasis between QTL, genotypic value of the individuals and heritabilities of quantitative traits can be readily estimated and analyzed.

Power, Precision and Accuracy of QTL mapping

The strength of the QTL can be determined based on the fraction of the total trait variance that it explains. Those, which explain over 20% of variance, are strong QTLs, traits controlled by such QTLs can be considered almost Mendelian. At the other extreme, weak QTL which explains 1% or less of the trait variance requires at least a thousand progeny to detect them with high power. Between those extremes are moderate QTL, which can be detected with crosses of reasonable size.

Carbon isotope discrimination ($\Delta^{13}C$) is currently the subject of intense in relation to crop drought resistance, as it its an effective integrated measure of long-term plant water use efficiency (Farquhar *et al.,* 1989b). Although Δ is a complex trait, Handley *et al.,* (1994) identified a major effect of barley chromosome 4 for Δ^{13} C in wheat-barley addition lines.

The composition of stable isotopes of plant carbon (δ^{13} C) was earlier shown to be an excellent indicator of WUE in Tomato and other species. Martin *et al* (1989) reported that δ^{13} could be satisfactorily predicted from three-restriction fragment length polymorphism (RFLP) that is genetic markers for discrete DNA sequences within the genome. An additive type of gene action was observed for all three RFLP markers, and for one of them there also was a significant non-additive component. Combining the δ^{13} C and RFLP technologies may aid in identifying genes associated with WUE and in mechanisms contributing to WUE. These technologies will also be useful for crop improvement.

Zhang and Xu, (2002) and Zhang, (2003), reported that in Wheat 2 QTLs controlling WUE, 10 QTLs significantly affecting per plant WUE (Total dry matter/ amount of water used per plant); 6 QTLs significantly controlling leaves and stem WUE (dry weight of roots /amount of water used per plant), 3 pairs of interaction QTL influencing root WUE were associated. There were gene clusters constituted by 2 or 4 QTLs linking closely together found on 1A, 3B, 4A and 6D chromosomes.

Nadaradjan *et al*., (2005) identified markers overlapping for WUE at whole plant level and $\Delta^{13}C$ in a DH population of rice. Five markers were found to be overlapping for both WUE and $\Delta^{13}C$ in experiment 1 and four in experiment 2, out of which marker OPZ1_1 was found to be consistent across the experiments.

Juenger *et al*., 2005, identified five QTL for flowering time and five QTL affecting $\delta^{13}C$ in RIL population of the cross Ler X Cvi in *Arabidopsis thaliana*. Two flowering QTL overlapped in genomic position with two $\delta^{13}C$ QTL.

ERECTA a putative leucine rich receptor like kinase was identified as a major contributor to a locus for $\Delta^{13}C$ Arabidopsis chromosome 2 (Masle *et al*., 2005). They also showed that this gene can modify transpiration efficiency by acting on epidermal and mesophyll development, stomatal density and the porosity of the leaves.

The genetic map of finger millet

Dida et.al.(2007) developed genetic map with 18 linkage groups in in an inter-sub specific F2 population from a cross between *E. coracana* subsp. *Coracana* cv. Okhale-1 and its wild progenitor *E. coracana* subsp. *africana* acc. MD-20using SSRs genic SSRs and AFLP markers. The maps span 721 cM on the A genome and 787 cM on the B genome and cover all 18 finger millet chromosomes

QTLs associated with root traits

Identifying molecular markers associated with roots would strongly augment the breeding efforts to both improve this trait and also to introgress the root traits with other agronomically desirable characteristics. To this end, several investigators have attempted to identify markers to the QTL regions conditioning root traits. Table 5 summarizes the literature in this area of research.

Much research on drought resistance in rice has focused on the root systems since it has been shown that drought avoidance is associated with the possession of a thick, deep root system (Yoshida and Hasegawa, 1982; Ahmadi, 1983; Loresto *et al*., 1983; Ekanayake *et al*., 1985 a & 1985 b).

Table 5 : QTLs associated with root traits in different crop species

Trait	Species	Type	QTL#	Reference
Root morphology	Rice	DHL	29	Li *et al.*, 2005
Root growth and Architecture	Arabidopsis	IL	13	Olivier *et al*., 2005
Root penetration ability & root thickness	Rice	RIL	20	Zheng *et al.*, 2000
Root length	Rice	DHL	2	Toorchi *et al*., 2002
Root traits	Rice	DHL	8	Kamoshita *et al.,* 2002
Root number, thickness & Dry weight	Rice	RIL	18	Champoux *et al*., 1995
Rootlength,No., thickness, penetration	Rice	RIl	28	Ali *et al*., 2000
Root traits and yield	Maize	F_3	11	Tuberosa *et al*., 2002
Total and penetrated root number, ratio	Rice	RIL	18	Price *et al*.,2000
Root growth traits	Rice	RIL	24	Price *et al*., 2002
Root morphological traits	Rice	DHL		Shashidhar *et al*., 1999
Root morphology and root distribution	Rice	DHL	39	Yadav *et al*., 1997
Root morphology, root cell length	Rice	F_2	24	Price and Tomos 1997
Root thickness, root penetration index	Rice	DHL	5	Zhang *et al*., 2001
Tiller and root number, penetration ability	Rice	RIL	39	Ray *et al*., 1996
Root thickness, root penetration index	Rice	DHL	12	Zheng *et al*., 2000
Yield and root traits under limited water	Rice	DHL		Venuprasad *et al*., 2002

Markers associated to some of the physiological traits in different crops.

Research experience in understanding the drought stress responses of plants, several plant physiological processes have been enumerated as important traits through which plants acquire tolerance or resistance to water stress situations. Most of these traits have also been shown to be quantitative in inheritance and hence efforts have been made to identify DNA markers linked with the QTLs influencing these traits. Mian *et al*., (1998) identified six putative independent RFLP markers associated with specific leaf weight in soybean. Out of these six markers four of the marker loci were consistent across the environments. They also identified three putative independent RFLP markers associated leaf size out of which was consistent across the environment.

Misawa *et al*., (2000) conducted QTL analysis for cold tolerance at the seedling stage by subjecting the rice plants to a temperature of 4 ^{o}C for 3 days. They identified 5 QTLs that were associated with low temperature response, one each on chromosome 1,9,and 11, two on chromosome 3. Andaya and Mackill, 2003 identified a major QTL designated as qCTSI2a, that was closely associated with cold induced necrosis and wilting tolerance in RILs of rice derived from the cross of M-202 X IR50. This QTL accounted for 41% of phenotypic variation. The relevant literature regarding the QTLs associated with different physiological traits is summarized in the table 6.

Ultimately the effectiveness of any MAS procedure will depend on the accuracy of the phenotypic classification of trait expression and the degree of linkage between the markers and the traits of interest. Although MAS may increase gain from selection when compared to phenotypic selection, marker utility in plant improvement programs will ultimately be determined by cost\unit information (Edwards and Page, 1994). Clearly, laboratory costs associated with MAS applications are decreasing and more effective and efficient molecular markers are being developed (Gu *et al*., 1995). This progress will make MAS more attractive and will foster its prudent implementation as a tool for plant breeding.

Table 6 : QTLs associated with various physiological traits

Trait	Species	QTL	Reference
Osmotic adjustment and dehydration tolerance	Rice	7	Lilly *et al*. 1996
Osmoregulation under drought	Wheat	1	Morgan and Tam 1996
Osmotic adjustment under drought	Barley	12	Teulat *et al*. 1998
Osmotic adjustment under drought	Rice	5	Zhang *et al*., 2001
Flowering parameters under drought, ASI	Maize	7	Ribaut *et al*., 1996
Plant height, ear number, days to silking, yield	Maize	5	Agrama and Moussa, 1996
Morphological and physiological traits	Rice	15	Hemamalini *et al*., 2000
Dehydration avoidance traits	Rice	17	Price *et al*., 2002
Grain yield and components under stress	Pearl millet		Yadav *et al*., 2002
Yield, biomass, Osmotic adjustment, roots	Rice	47	Chandra Babu *et al*., 2003
Abscisic acid concentration	Wheat	1	Quarrie *et al*., 1994
Leaf ABA concentration	Maize	16	Tuberosa *et al*., 1998
Leaf rolling and stomatal conductance	Rice	8	Price *et al*., 1997
WUE, RWC, Dry matter, carbon discrimination	*S. scabra*	36	Thumma *et al*., 2001
Photosynthesis and water status traits	Sunflower	19	Herve. *et al*. 2001
Dry mass, Na^+, K^+ uptake, concentration and ratio	Rice	25	Koyama *et al*., 2001
Na+ K+ absorption	Rice	2	Bonilla *et al*., 2002
Growth and germination	Arabidopsis	11	Quesada *et al*., 2002
Cold tolerance at the booting stage	Rice	3	Takeuchi *et al*., 2001
Chilling tolerance for spikelet sterility	Rice	2+	Andaya & Mackill, 2003
Heat tolerance in grain filling	wheat	2	Yang *et al*., 2002
Stay green, chlorophyll content	Sorghum	13	Xu *et al*., 2000
Stay green under drought	Sorghum	5	Tao *et al*., 2000
Stay green, chlorophyll content	Sorghum	7	Crasta *et al*., 1999
Stay green, pre-flowering drought stress	Sorghum	9	Kebede *et al*., 2001
Stay green	Sorghum	4	Sanchez *et al*., 2002
Stay green; chlorophyll content	Rice	1	Cha *et al*., 2002

Marker Assisted Selection

Selecting plants in a segregating progeny that contain appropriate combinations of genes is a critical component of plant breeding (Ribaut&Betran, 1999;Weeden et al., 1994). Moreover, plant breeders typically work with hundreds or even thousands of populations, which often contain large numbers (Ribaut & Betran, 1999; Witcombe & Virk, 2001). ‘Marker-assisted selection’ (also ‘marker-assisted breeding’ or ‘marker-aided selection’) may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods. Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Michelmore, 1995; Ribaut et al., 1997; Young,1996)

Material & Methods

III. MATERIAL AND METHODS

The present investigation involves the development of SSR markers in finger millet and their validation in mapping population (Recombinant inbred lines) developed from two contrasting parents (IE 2912 and IE 2885) for drought traits and molecular characterization of 150 RILs to identify the prominent DNA markers associated with some drought traits.

3.1 Development/Isolation of SSR markers

Three approaches were followed to develop/outsource the markers and utilize them for the present study, i.e., (a) development of genomic SSRs by precloning enrichment strategy, (b) screening with rice and finger millet SSRs from public domain and (c) finally to develop genic SSRs.

3.1.1 Genomic SSRs: The pre-cloning enrichment (selective hybridization) strategy was followed to fish out microsatellites from fingermillet genome. This strategy employs the whole pool of digested DNA by subjecting them to repeat oligomer hybridization, thereby leading to selective enrichment.

3.1.1.1 The steps followed in the microsatellite isolation by pre-cloning enrichment (selective hybridization) strategy are given below :

I. DNA extraction

II. Restrict digestion of extracted DNA

III. Linker ligation to DNA fragments

IV. Enrichment of microsatellite repeats

V. Capturing of repeat regions from enrichment PCR product

VI. Cloning and sequencing of repeat containing DNA fragments

VII. Sequence analysis and primer designing to specific microsatellite repeats

I. DNA extraction

The young leaves of one of the parents, IE2912 were collected and frozen in liquid nitrogen for DNA extraction. DNA was extracted by CTAB method explained by Saghai - Maroof *et al.* (1984).

II Restrict digestion (using RsaI or Hae III)

The isolated genomic DNA (4 µg) was restrict digested with RsaI (1 unit) in 1X buffer to get approximately 500bp fragments in a 50µl reaction mixture at 37^{0}C overnight. An aliquot (4-5µl) of the digested sample was electrophoresed on 1.0% agarose gel with a 1kb ladder.

III Linker ligation to DNA fragments (super SNX linker ligation)

The aim here is to ligate a double stranded linker onto each end of each digested DNA fragment, so that the linker provides the primer binding site for subsequent PCR steps. They as well provide sites to ease cloning of the fragment into the vectors used subsequently. The linker is therefore compatible with the restriction sites in the vectors multiple cloning site (MCS). The super SNX also incorporates a GTTT pigtail to facilitate non template ‘A’ addition by *Taq* DNA polymerase during PCR which can be used for T/A cloning (pTZ57R/T). The linkers used are given below:

Super SNX24 forward- 5’ GTTTAAGGCCTAGCTAGCAGAATC

Super SNX24+4p reverse- 5’ pGATTCTGCTAGCTAGGCCTTAAACAAAA

(a) Preparation of double strand super SNX linkers

The double stranded linkers were made by heating equimolar concentration of both forward and reverse linkers at 95^{0}C in presence of 100mM NaCl, and allowed to cool gradually to room temperature. These double stranded linkers were ligated to RsaI digested genomic DNA. This ligation reaction mixture contained 4 µg of digested DNA, 70µM of linkers in presence of 5µM ATP, 10 units of DNA ligase, 1 unit of Xmn1 and 1 X buffer. Finally the ligation was carried out at 16^{0}C overnight.

(b) PCR of the linker ligated DNA

To ensure that the linker ligation has worked, PCR of linker ligation mix was performed in a 25µl reaction as follows: 2µl of ligated DNA mix as template, 10µM of SNX 24 forward primer, 150µM of dNTPs, $MgCl_2$, 1 unit of *Taq* DNA polymerase and 25 µg/ ml of BSA, in 1x *Taq* DNA polymerase buffer. The PCR cycling conditions were as follows: 95^0C (initial denaturation) for 2 min followed by 20 cycles of denaturation at 95^0C for 20sec, annealing at 60^0C for 20sec. and extension at 72^0C for 1 min 30sec.Approximately 5µl of PCR product was electrophoresed on 1.2% agarose gel to check if the linker ligation was successful, using 100bp ladder as a marker.

IV Enrichment for microsatellite containing genomic DNA fragments

The aim here is to remove the unwanted DNA fragments and capture the repeat containing DNA fragments (microsatellites) using biotinylated complementary microsatellite oligos (probes), The biotinylated oligos used for enrichment were: $(CA)_{17}$, $(AG)_{16,}$ $(AGC)_8$, $(AGG)_{8,}$ $(AAC)_{14,}$ $(ATC)_{14,}$ $(AAG)_{14,}$ $(ACGC)_{5,}$ and $(ACCT)_{8.}$

The materials required for this step are

a) Streptavidin beads (washed twice in tris EDTA buffer and twice in 1x hybridization solution).

b) 2x hybridization solution (2x hyb) - 12x SSC, 0.2% SDS (warmed)

c) 1x hybridization solution (1x hyb) – 6x SSC, 0.1%SDS (warmed)

d) Washing solution- 2x SSC, 0.1% SDS (warmed)
1x SSC, 0.1% SDS (warmed)

e) Biotinylated oligos (individual or mixture of 3' biotinylated oligos)

*(SSC-Sodium Saline Citrate, SDS-Sodium Dodecyl Sulphate)

The enrichment of microsatellites was carried out in final volume of 50µl containing 25 µl 2x hybridization solution, 10 µl equimolar biotinylated microsatellite oligos and 2µg of linker ligated DNA. The PCR amplification was carried out using an

Eppendorf Master Cycler gradient. The programme consisted of the following steps; 99 cycles of amplification follows 95^0C/5 min, 70^0C/5 sec, 68.8^0C/5 sec, 68.6^0C/5 sec with step down of 0.2^0C for every 5 sec until it reaches 50^0C and maintained at 50^0C for 10 min, followed by 20 cycles consisting of 49.5^0C/5 sec with step down of 0.5^0C every 5 sec until it reaches 40^0C/5 sec and finally held at 15^0C. The touch down PCR conditions facilitate the microsatellite probes to hybridize with complimentary DNA repeat fragments (i.e., expectantly long prefect repeats) when the reaction mixture is at or near the microsatellite probes melting temperature.

V Capturing of repeat regions from enrichment PCR product

After enrichment reaction, to capture microsatellite repeats the following steps were performed.

1) The streptavidin beads were washed twice with Tris-EDTA (TE) buffer and twice in the hybridization solution as follows: 2x hyb. (12x SSC, 0.2% SDS), 1x hyb (6x SSC, 0.1% SDS) and washing solution (2x SSC, 0.1% SDS, 1x SSC, 0.1% SDS)
2) To the enrichment reaction mix, 50µl of washed streptavidin Dynabeads were added and transformed into 1.5 ml eppendorf tube containing 250µl of TE and mixed well.
3) These beads were captured under the magnetic particles collecting (MPC) unit and again these beads were washed with TE and 1x hyb solution. Finally these beads were incubated at room temperature after resuspension in 150µl of 1x hyb solution kept on rotator with low speed for 30 min.
4) After incubation, the supernatant was removed and beads were collected under the magnetic field. These beads were again washed twice with washing solution at 45^0C to 50^0C.
5) After washing 200µl TE was added, vortexed and incubated at 95^0C for 5 min. Then the beads were captured under the magnetic unit and supernatant containing enriched fragments (i.e., “**GOLD**”) were collected into the new tube.

6) To the microsatellite containing supernatant, 22µl of 3M NaOAc (Sodium acetate) and 444 µl of 95% ethanol was added, mixed well and kept on ice for 15 min and then centrifuged at 12,000rpm for 10 min

7) Supernatant was discarded and 0.5 ml of 70% ethanol was added, centrifuged for 1 min, again the supernatant was discarded and pellet was resuspended in 25 µl of TE (**Pure Gold**)

PCR recovery of enriched DNA

To recover the enriched pure gold DNA fragments, PCR was carried out in a 25 µl reaction containing 1x PCR buffer, 0.5 µM super SNX Forward primer, 150 µM dNTPs, 2.0 mM $MgCl_2$, 25 µg/ml BSA, 1 U *Taq* DNA polymerase, and 20 ηg of pure gold DNA.

The DNA amplification was carried out using a PTC 100 (MJ Research) thermal cycler, programmed with 3 min at 94^0C for initial DNA denaturation, followed by 25 cycles of 94^0C for 20 sec, 60^0C for 20 sec, 72^0C for 1.5 min, final extension at 72^0 C for 30min.

VI Cloning and sequencing of repeat containing DNA fragments

(a) Ligation of enriched DNA fragments into pTZ57 R/T vector

Purified PCR product (50ng) was ligated to PTZ57R/T linearized vector (100 ng) using 1U of T4 DNA ligase over night at 16^0C in a 10 µl reaction volume. Around 2.5 µl of ligated mix was used for bacterial transformation in DH5α competent cells (Sambrook *et al.,* 1989). The transformed bacteria were plated on ampicillin selection with X-gal for blue white selection. White colonies were screened by colony PCR using M13 forward and reverse primers (3 µM), 100 µM dNTPs, 2 mM $MgCl_{2,}$ 1 U of *Taq* DNA polymerase and 1X PCR buffer in 15 µl reaction volume, at annealing temperature of 57^0C and final extension at 72^0 for 2 min. The PCR products was resolved on 0.8% agarose gel, using 100bp ladder and the inserts ranging from 500-1000bp were selected and again amplified in a 50 µl reaction and purified for sequencing, using the PCR purification kit (SIGMA).

(b) DNA sequencing

The purified PCR products were sequenced with nested M13 forward and reverse primers using ABI prism sequencer (Delhi University, South Campus, New Delhi).

(c) Analysis of the sequence data

The sequences obtained were analyzed for the presence of microsatellite regions using the web-based software –"Tandem Repeat Finder".

VII Primer designing and PCR standardization

(a) Primer designing

Clones containing >15bp microsatellite region were selected for primer designing using the FastPCR software programme. Primers were designed in the flanking region of microsatellites and synthesized as desalted oligos (SIGMA) PCR standardization was carried out using the genomic DNA as template to arrive at the optimum annealing temperature for each primer synthesized.

(b) Optimization of annealing temperature for microsatellite primers

The PCR amplifications were carried out in 15µl reaction volume containing; genomic DNA (10ηg/µl), PCR Buffer (1X), $MgCl_2$ (2.5mM), 2mM dNTPs, 2µM forward and reverse primers and 1 unit *Taq* DNA Polymerase. Amplification was performed in a Eppendorf Master Cycler Gradient, programmed for initial denaturation at 94^0 C for 5 minutes, followed by 30 cycles of denaturation at 94^0 C for 60sec, gradient annealing temperature (± 5^0 C of oligos Tm) for 30sec, primer extension at 72^0 C for 1 min and a final extension of 10 min at 72^0 C. The amplified products were resolved on 0.8% agarose gel in 1x TBE (40mM Tris-acetate, 1 mM EDTA pH 8.0) buffer at 90 volts for 2 h. The gels were documented using HeroLab Gel Documentation system.

3.1.2 Development of genic SSRs from stress specific finger millet EST

Simple sequence repeats developed from the existing finger millet EST sequences could act as genic SSRs a powerful marker system as these are present in coding region of the genome. To develop genic SSRs, available dehydration stress specific EST library constructed by subtractive hybridization at the Department of Crop Physiology, UAS Bangalore was used. These EST sequences were further annotated for the presence of microsatellite regions by the web based programme-Tandem Repeat Finder.

3.2 Physiological characterization of finger millet

3.2.1 Experiment I

3.2.1.1 Development of mapping population

Recombinant Inbred Lines (RILs) were developed by crossing two Indo-African accessions of Finger Millet (*Eleusine coracana* Geartn. L.) following the Single Seed Descent Method. The mapping population was developed by Dr.Shailaja Hittalamani, Department of Genetics and Plant Breeding. The genotype IE-2912 is a well characterized source of blast and drought traits (Table 7)

3.2.1.2 Establishment of seedlings in pots

During Kharif 2005, instead of normal direct sowing, transplanting was taken up for which individual recombinant inbred line along with parents was sown in individual pots. RILs were grown upto 25 days in the pots.

3.2.1.3 Transplanting of seedlings in specialized root structures

After 25 days of sowing the seedlings were transplanted in the specialized root structures with a dimension of 10X60 sq.ft. (Plate 1) in three replications and the individual lines were replicated in a row in each structure. Plant nutrition was taken care by providing N:P:K (50:40:25 Kg/ha recommended dose) in two split doses. The transplanted RILs were evaluated for physiological traits such as WUE, root traits, total dry matter, $\Delta^{13}C$.

Table 7 : The salient features and pedigree details of the parents of mapping population

Sl. No	Name of the parents	Salient features
1	IE-2912	It is resistant to neck blast and drought
2	IE-2885	It is susceptible to neck blast and drought

Recombinant Inbred Lines (RILs) were developed by crossing two Indo-African accessions of Finger Millet (*Eleusine coracana* Geartn. L.) following the Single Seed Descent Method. The genotype IE-2912 is a well characterized source of blast and drought traits

Plate 1 : RILs grown along with parents in specialized root structures for phenotyping

Two parents IE 2912 and IE 2885 used in this study were selections from *Elusine coracana* contrasting for drought and neck blast resistance. The parent IE 2912 is resistant for neck blast and drought whereas IE 2885 is susceptible for neck blast and drought. Using these two contrasting parents 150 RILs (recombinant inbred lines) were developed and these RILs were used for both phenotyping and genotyping

3.2.1.4 Morpho physiological traits

3.2.1.4.1 Measurement of Leaf Area

A sample of 5 leaves from one plant was taken and their area was determined by measuring the L X B of each leaf. The ratio of leaf area to the leaf dry weight was computed as Specific leaf Area. The remaining leaves of a plant were separately oven dried. The dry weight was multiplied with actual SLA to arrive at the total plant leaf area.

Leaf area was measured by Length X Breadth method

Leaf Area = Length of the leaf in cm X Breadth of the leaf in cm

SLA = leaf area in cm^2/ leaf dry weight in g

3.2.1.4.2 Total Biomass (BM)

Entire plants including roots were harvested and oven dried at 70^oC for three to five days. The total biomass accumulated was calculated expressed as gram per plant.

3.2.1.4.3 Root length

The roots were separated from the plants and the root length was recorded using the graduated scale.

3.2.1.4.4 Root Volume

A known volume of water was taken in a graduated beaker, into this the separated roots were immersed and then the increase in the volume of water was recorded which actually represents the actual root volume.

3.2.1.4.5 Tiller number per plant and Plant Height

At the time of harvest, numbers of tillers were counted from the plants and the plant height was measured and expressed in centimeter

3.2.1.4.6 Measurement of chlorophyll content using SPAD meter (SCMR)

SCMR (SPAD chlorophyll meter reading) values which are an indication of chlorophyll status in the leaf was measured using SPAD meter. Portable SPAD meter was clamped onto the leaf at different positions as well as on different leaves (3rd, 5th, 7th leaf from top) of the plant and the SPAD reading was measured. The mean of SCMR reading was taken out in the end and presented as average SPAD values.

3.2.1.4.7 Moisture Retention Capacity (MRC)

Five leaves from each RIL were detached and immediately fresh weight was recorded, subsequently fresh weight of leaves of all the RILs was recorded at every successive 20 minute interval upto three hours. After three hours, leaves were kept for oven drying for 2-3 days at 60^0 C. Using both fresh and dry weights, MRC was calculated using the formula:

MRC= {(FW1-DW)/ (FWi-DW)} X100

FWi - Fresh weight immediately after harvest in gram (initial fresh weight)

FW1 - Weight at a particular hour after harvest in gram

DW - Oven dry weight in gram

3.2.2 Stable isotope studies

3.2.2.1 Determination of genetic variability in WUE based on Carbon Isotope Discrimination ($\Delta^{13}C$)

During the fixation of carbon by photosynthesis, the naturally occurring heavy stable isotope, ^{13}C, is discriminated against ^{12}C. Therefore, plants have a smaller ratio of ^{13}C to ^{12}C than does the CO_2 of air that feeds them. Farquhar *et al*., (1982) developed the theory, which predicted that this discrimination would be least in those C_3 plants that fix the most carbon per unit amount of water transpired i.e. in those that have the greatest water use efficiency. Ever since then, $\Delta^{13}C$ technique has been widely adopted as a powerful surrogate estimate of WUE.

3.2.2.2 Determination of $\Delta^{13}C$ in leaf samples

The Carbon Isotope Discrimination ($\Delta^{13}C$) was determined with an objective to study how $\Delta^{13}C$ is associated with the observed genetic variability in WUE in finger millet RILs.

1. Third fully expanded leaves that were developed during the experimental period were collected (5-6 for each genotype)
2. Incubated at 80°C for 3 days to completely dry the samples.
3. Dried samples were finely powdered in a mortar and pestle. Care was taken to prevent mixing of different samples by washing the pestle and mortar with alcohol after grinding each sample.
4. One gram of powdered leaf sample was put in glass vial and properly labeled.

The $\Delta^{13}C$ of the leaf samples was determined using Continuous-Flow Isotope Ratio Mass Spectrometer (IRMS) at the National Facility for stable isotopes studies in Biological Sciences, Department of Crop Physiology, UAS, Bangalore. The details of the methodology are described below:

3.2.2.3 Mass spectrometric analysis

Carbon isotopic discrimination is measured as the deviation of the molar ratio between ^{13}C and ^{12}C from an international standard –PDB (Pee Dee Belmnite). Carbon isotopes are fractionated due to diffusion through stomata denoted as “a” and at the carboxylation site by RuBisCO denoted as “b”. The total isotope discrimination is the deviation of these fractions from the isotopic ratio in air. Therefore the discrimination is $\Delta^{13}C$ computed as follows.

$$\boldsymbol{\Delta^{13}C = \delta a - [a + (b - a)\, Ci/\, Ca]}$$

where a = 4.4 ‰, b = 29 ‰, Ci and Ca respectively are the CO_2 concentration inside the leaf and in the ambient air.

Alternatively, $\Delta^{13}C$ is also computed as the deviation of isotopic composition of the plant organic sample from that of the ambient air, as follows:

$$\Delta^{13}C\ \mathbf{(‰)} = \frac{\delta a - \delta p}{1 + \delta p/1000}$$

where,

$\delta_a = \{(R_{air} - R_{PDB})/ R_{PDB}\}$ X 1000

$\delta_p = \{(R_p - R_{PDB})/ R_{PDB}\}$ X 1000

$R = {}^{13}C/{}^{12}C$

The IRMS installed at our center works on the continuous flow basis interfaced with an elemental analyzer (CN1112, CarloErba (CE), Italy) for sequential combustion of biomass samples to generate CO_2 gas.

Finely powdered leaf samples were accurately weighed in the range of 0.8 to 1.2 mg into silver capsules. The crimped capsules with the sample were placed sequentially in the carousel of the Autosampler. The samples were dropped at precise times along with an injection of pure O_2 into the oxidation reactor.

The Combustion (oxidation) reactor contains chromium oxide and silvered cobaltous-cobaltic-oxide in a quartz column heated to 1080^{o}C. The biomass is completely oxidized to produce CO_2, N_2O and H_2O. These gases were swept into the reduction furnace using helium carrier gas (purity 99.995%). The reduction furnace contains reduced copper in quartz tubes heated to 680^{o}C. In this reaction, the N_2O is reduced to N_2 and the excess O_2 is absorbed. The resultant gases are then flushed through scrubbers to trap water. The pure CO_2 and N_2 gases are then passed through a GC column (5^{o}A molecular sieve). The N_2 gas elutes faster through the GC and hence gases can be effectively separated before introduction into the ion source of the IRMS. At the ion source, CO_2 is ionized by electron impact ionization to produce molecular radicals (CO_2). These CO_2 radicals are accelerated under the influence of high voltage potential 3000 volts through a strong magnetic field. When accelerated, radicals pass through a strong magnetic field, the radicals are deflected with the radius of deflection being proportional to the molecular mass of the radicals. Faraday cups collect these deflecting $^{12}CO_2$ and $^{13}CO_2$ and the signal is amplified and transmitted to the computer.

3.2.3 Statistical analysis

The data obtained from experiments were analyzed using statistical software packages like MSTATC and MS EXCEL, etc.The genotypic variability of physiological traits were assessed using Analysis Of Variance as per Fisher's method. The level of significance was tested at 0.05 and 0.01 probability level in 'F' test. The genotypic means were compared with the critical difference values. This analysis was performed using MSTATC.

3.2.4 Phenotypic and genotypic coefficient of variations

The phenotypic and genotypic coefficients of variations were computed as per Burton and Dewane (1953).

$$\text{PCV \%} = (P/X) \times 100$$

$$\text{GCV \%} = (G/\overline{X}) \times 100$$

where,
P = Phenotypic standard deviation
G = Genotypic standard deviation
X = Grand mean of the character
PCV = Phenotypic coefficient of variation
GCV = Genotypic coefficient of variation
GCV and PCV were classified as given below.

0 –10 % - low
10- 20% - moderate
20% and above – high

3.2.5 Heritability

Broad sense heritability was estimated using the formula (Hanson *et al.*, 1956).

$$H\ \% = (V(g) / V(p)) \times 100$$

Where,

H = broad sense heritability

V(g) = Genotypic variance

V(p) = Phenotypic variance

Heritability was categorized into following three classes.

0-30 % - low heritability
30-60% - medium heritability
60% and above- high heritability

3.2.6 Genetic advance

Genetic advance was calculated by the formula given by Johnson *et al*, 1955

$$GA = H \times \sigma p \times k$$

where,

H is the broad sense heritability

σ P = phenotypic standard deviation

k = selection differential which is 2.06 at 5% intensity of selection (Lush, 1949).

3.2.7 Genetic advance as per cent mean

GA as per cent mean = (GA /X) x 100

where GA = Genetic advance

Genetic advance as per cent means (Johnson *et al*., 1955) are given below:

0-10% - low
10-30% - moderate
20% and above – high

3.3 Experiment II

3.3.1 Establishment of seedlings in pots and transplanting of established seedlings in specialized root structures

The second phenotyping experiment was carried out in Kharif 2007, wherein, transplanting was taken up for which individual recombinant inbred line along with parents was sown in individual pots. RILs were grown upto 25 days in the pots. After 25 days of sowing the seedlings were transplanted in the specialized root structures with a dimension of 10X60 sq.ft. in the same manner as in first experiment with three replications. Plant nutrition was taken care by providing N:P:K (50:40:25 Kg/ha recommended dose) in two split doses. The transplanted RILs were evaluated for physiological traits such as leaf traits, root traits, total dry matter etc.

3.3.2 Morpho physiological traits

3.3.2.1 Total Biomass (BM)

Entire plants including roots were harvested and oven dried at 70°C for three to five days. The total biomass accumulated was calulated expressed as gram per plant.

3.3.2.2 Root length

The roots were separated from the plants and the root length was recorded using the graduated scale.

3.3.2.3 Root Volume

A known volume of water was taken in a graduated beaker, into this the separated roots were immersed and then the increase in the volume of water was recorded which actually represents the actual root volume.

3.3.2.4 Tiller number per plant and Plant Height

At the time of harvest, numbers of tillers were counted from the plants and the plant height was measured and expressed in centimeter

3.4 Molecular Characterization of Recombinant Inbred Lines (RILs)

The young leaves of the RILs along with the parents were collected in liquid nitrogen for DNA extraction. DNA was extracted from all the 150 RILs using CTAB method explained by Saghai - Maroof *et al.,* (1984).

3.4.1 Protocol for extraction of genomic DNA

One gram of fresh young leaf sample was taken in a mortar and powdered well using liquid nitrogen.

↓

The ground tissue was transferred to a 50 mL sterile centrifuge tube by adding 15mL of CTAB buffer (Extraction buffer- 2%C-TAB, 1.4 M NaCl, 20 mM EDTA (Disodium) & 10mMTrisbase (pH 8)). Then 50 μL of β- mercaptaethanol was added to each tube and the extract was mixed well by inverting the tubes several times. The tubes were incubated in a water bath maintained at 65 ^{0}C for an hour with constant stirring at an interval of 15 minutes.

↓

After an hour, 15 mL of chloroform isoamyl alcohol (24:1) was added to the incubated sample and mixed well by inverting. The tubes were then centrifuged at 6000 rpm for 20 minutes.

↓

The aqueous upper phase was carefully transferred using 1mL cut tips into fresh sterile centrifuge tubes. To this supernatant, 0.7 volumes (10.5mL) of cold isopropanol was added.

↓

The tubes were carefully inverted and kept for 5 min on ice. The precipitated DNA was then centrifuged at 6000 rpm for 20 minutes and sedimentation of DNA as a pellet was seen.

↓

Further the supernatant was decanted gently and the tubes were inverted on a clean filter paper.

↓

The pellet was washed twice by suspending in one mL of 70% ethanol for 5 to 10 minutes and centrifuged at 6000 rpm for two minutes.

↓

Ethanol was drained off slowly and the pellet was vacuum dried in a dessicator for 5 to 10 minutes. The pellet was then dissolved in 500 µL of TE buffer by flicking the tubes. (TE buffer = 0.1mM Tris + 0.05mM EDTA)

↓

To remove the RNA, 5µL of RNase (10mg/mL) was added into the DNA solution and incubated at 37°C in a water bath for 1 hour.

↓

Again the DNA solution was cleaned by washing with equal volume (500 µL) of phenol: choloroform:Isoamyl alcohol (25:24:1) by invert mixing several times and centrifuging at 6000 rpm for 15 minutes to separate the two phases.

↓

The aqueous upper phase was transferred into a clean 1.5 mL eppendorf tube and two volumes of absolute ethanol was added to precipitate the DNA.

↓

The pellet was washed with 70% ethanol twice and dissolved in 500 µL TE.The extracted DNA was quantified using both Nanodrop DNA quantifier and also by electrophoresis on 0.8% agarose gel.

3.4.2 DNA purity assessment

The purity of genomic DNA was quantified by Nanodrop DNA quantifier (JH, Bio), measured the absorbance ratio of 260/280 nm. Pure DNA preparations showed a ratio of 1.8 to 2.0.

3.4.3 DNA quantification on agarose gel

Agarose gel submersible electrophoresis is used to separate the DNA based on size (0.1 to 2.5 kb). Agarose gel electrophoresis is usually conducted in a submerged horizontal tray. The tray is chosen in such a way that its length should allow required band resolution. Genomic DNA usually is electrophoresed on 0.8 to 1% agarose.

3.4.3.1 Preparation of agarose gel

0.8g of agarose was weighed and taken in a clean 100mL conical flask. To this 100 mL of 1x TBE buffer was added. TBE buffer (0.89MTris base, 0.02MEDTA, 0.89M Boric acid: pH = 8)

↓

Agarose was dissolved by heating. After agarose completely melts, it was cooled to 60^{o}C and 3.5 μL of ethidium bromide was added (10mg/mL) and mixed.

↓

The ends of gel casting trays were sealed with tapes. Agarose was poured; comb was inserted and allowed to solidify.

↓

After solidification the tape on either side of the tray was removed and the gel was immersed in electrophoresis tank having 1x TBE buffer. Then the comb was removed.

↓

To 5 μL of DNA samples, 3μL of 6x loading buffer was added, mixed well and loaded into the well. 3μL of standard uncut Lambda DNA was used as marker. (Loading buffer or tracking dye 6x – 40% sucrose, 0.025% bromophenol blue, 0.25% xylene cyanol)

↓

Electrophoresis was carried out at 50V for 2 to 3 hours until the bromophenol blue dye migrated two-third of the gel. The gel tray was removed and the gel was observed under UV transilluminator and documented using Herolab Gel Doc system (Belgium). The quantity of the DNA was determined based on the intensity of the band relative to lambda uncut band.

3.5 Molecular Markers/ DNA Markers

3.5.1 Simple sequence repeats analysis (SSR)

Microsatellites are simple sequence repeats (SSRs) of 1-6 nucleotides. They appear to be ubiquitous in higher organisms, although the frequency of microsatellites varies between the species. They are abundant, dispersed throughout the genome and show higher levels of polymorphism than other genetic markers. These features, coupled with their ease of detection, have made them useful molecular markers.

3.5.1.1 Components of PCR

The reaction mixture was set up in sterile 0.2mL microfuge tubes. The reaction mixture volume per one reaction is as follows.

10x Taq Polymerase buffer	1.5µL
2 mM dNTPs	1.5µL
$MgCl_2$	0.5µL(2mM)
Forward primer (1pMole/µL)	2.0µL
Reverse primer (1pMole/µL)	2.0µL
Taq Polymerase (1U)	0.30µL
Template DNA (20-30ng)	2.0 µL(10ng/µL)
Sterile water	5.2µL
Total volume	**15.0 µL**

3.5.1.2 Standardization of annealing temperature for SSR primers

The annealing temperature was standardized by setting gradient PCR. In this method, different annealing temperatures (Tm+/-5^{0}C) were set to each block and amplification was carried as per below mentioned reaction conditions. After standardization of annealing temperature, the parents and progeny were amplified using the same protocol.

3.5.1.3 SSR cycling condition

Initial denaturation 5 min at 94°C

$$\left.\begin{array}{l}\text{Denaturation 1 min at 94°C}\\ \text{Annealing 30 sec at 55-65°C (primer specific)}\\ \text{Extension 1min at 72°C}\end{array}\right\}\quad \text{30 cycles}$$

Final extension 8 min at 72°C

Agarose gel (0.8%) was prepared as described earlier and the PCR amplified products were electrophorased on agarose gel. Primers that amplified at different gradient annealing temperatures were recorded.

3.5.2 Parental Polymorphism

Once the primers were standardized for their annealing temperatures, such primers were used for parental polymorphism. Initially the parents were screened to get the polymorphic primers. Subsequently these polymorphic primers were used to screen the RILs

3.5.3 Genotyping

Primers which revealed polymorphism between the parents were used for genotyping in the RILs(Recombinant Inbred Lines). Genotyping basically involves extracting the genomic DNA from all recombinant inbred lines, screening them using polymorphic primers by PCR and resolving the PCR products on high per cent agarose gel and observing the segregation pattern of specific markers in mapping population.

3.5.4 Random amplified polymorphic DNA (RAPD) analysis

3.5.4.1 PCR amplification of DNA with random primers

A total of 320 random primers were screened between the parents to identify the polymorphism. These primers were obtained from OPERON technologies. The procedure for RAPD analysis is as follows.

3.5.4.2 Components of RAPD PCR

The reaction mixture was set up in sterile 0.2mL microfuge tubes. The reaction mixture volume per one reaction is as follows.

10x Taq Polymerase buffer	1.5µL
2 mM dNTPs	1.5µL
$MgCl_2$	0.5µL(2mM)

Random primers (1pMole/μL)	5.0μL
Taq Polymerase (1U)	0.3μL
Template DNA	3.0μL(10ng/μL)
Sterile water	3.2μL
Total volume	15.0 μL

Reaction mixture was prepared in multiples, if more than one sample is planned for a primer and template DNA was added at the end to each tube separately.

3.5.4.3 Random primer Amplification condition

PCR tubes with reaction mixture were spun briefly for a minute, placed in the thermo-cycler and the lid of thermocycler was closed. Amplification was carried out in a thermocycler (Eppendorf-AG, Germany), with the following amplification conditions.

Initial denaturation 5 min at 94°C

Denaturation 1 min at 94°C

Annealing 30 sec at 35°C } 35 cycles

Extension 45 sec at 72°C

Final extension 5 min at 72°C

The PCR products were analyzed by agarose gel electrophoresis (1.5 %) The primers showing polymorphism between the parents were selected for reconfirmation. The reconfirmed polymorphic primers were used for screening the 152RILs.The-amplified products were electrophoresed and genotyped for the segregating bands.

3.6 Map construction by MAPMAKER/EXP 3.0

A molecular linkage map was constructed using MAPMAKER / EXP 3.0 with a threshold of 3.0. The Mapmaker is an interactive computer package for constructing genetic linkage map and for mapping genes underlying complex traits. It consists of twin programmes namely MAPMAKER/EXP and MAPMAKER/QTL. MAPMAKER/EXP facilitated map construction. It performs full multipoint linkage analysis from primary data for dominant, recessive and co dominant marker in F2, backcross, F_3, RILs and DHs.

Based on Lander Green algorithm, it calculates the best map for any given order of loci (Lander *et al*., 1987). The map was developed based on 95 RAPD marker loci, and 15 SSR marker loci using 150 RILs.

3.7 Association of marker / QTL for physiological traits

3.7.1 Single marker analysis (SMA)

Association between various markers and the phenotypic performance of the individuals for various traits was performed using linear regression analysis in MS-EXCEL. Different physiological traits were treated as dependent variables and the various molecular marker genotypes as independent variables.

The proportion of the total phenotypic variation explained by each marker associated with the QTL (R^2) was calculated as the ratio of the sum of squares examined by the marker locus to the total sum of squares at a probability level $p < 0.1$.

Results

IV. RESULTS

Though a C4 species, growth and productivity of finger millet got significantly affected by drought stress. Enhancing drought tolerance through trait-based breeding approach is the most plausible approach. Effective trait introgression can be achieved through the use of tightly linked DNA markers. Despite the enormous potential of molecular breeding, lack of genetic as well as genomic resources in finger millet has been the most important constraint in initiating molecular breeding programs. In this investigation we developed genomic resources of SSR markers and utilized them for genetic characterization of a recombinant inbred line (RIL) population. Results obtained in several of these experiments are described in this chapter.

4.1 Development of SSR markers from finger millet genomic DNA

Microsatellites or SSRs (simple sequence repeats) are short tandem repeats of DNA that are widely dispersed in all eukaryotic genomes. Microsatellites are generally co-dominant and highly polymorphic. This high level of detected polymorphism mainly results from a variable number of repeats, primarily owing to replication slippage, although unequal crossing over might also be involved in the genome. These markers are increasingly being used to assess germplasm diversity, linkage analysis and molecular breeding. In this study, 323 novel finger millet specific microsatellite markers were developed from a partial genomic library.

4.1a Restriction digestion and Linker ligation to digested DNA fragments

The genomic DNA isolated from leaves of finger millet variety IE 2912 was digested with RsaI enzyme to obtain fragments of approximately 500 bp (Plate 2a). The fragmented DNA was ligated with double stranded super SNX linkers, which provides the primer-binding site for the subsequent PCR steps and facilitates cloning into vectors. To ensure the linker ligation, PCR of linker ligation mix was performed and 5µl of PCR product was resolved on 1.2% agarose gel, using 1kb ladder as a standard (Plate 2b).

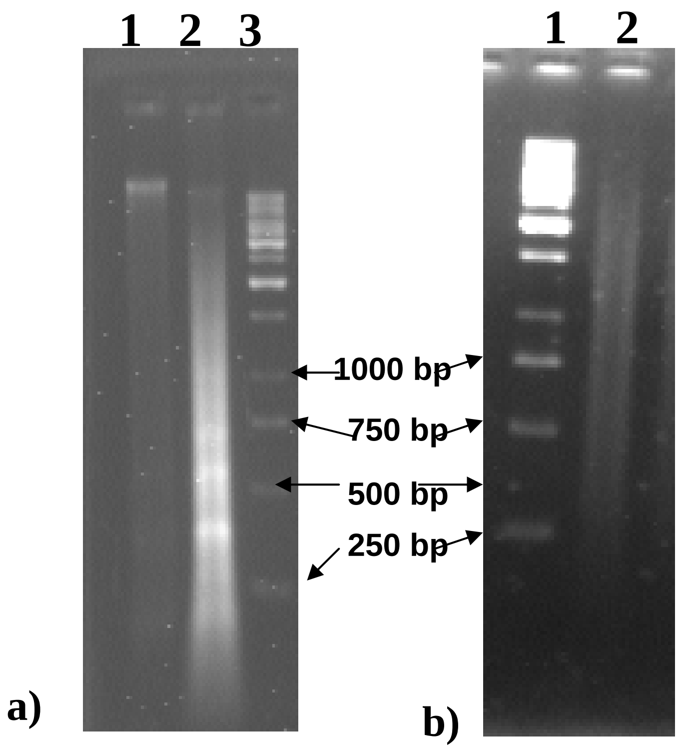


Plate 2 : Restriction digestion of genomic DNA and Linker ligation to digested DNA fragments

a) The genomicDNA isolated from leaves of one of the parents of mapping population i.e.,IE2912 (lane1) was digested with *RsaI* enzyme (lane2) to fragment the DNA into approximately 500bp; Lane3- 1kb marker

b) The digested fragments were further ligated with double strand super SNX linkers. to ensure linker ligation, the ligated DNA was amplified withSNX24 forward primers (lane2); Lane1- 1kb marker

4.1b Enrichment of microsatellites containing genomic DNA fragments

The enrichment reaction was carried out to enrich the repeats from linker ligated PCR product and removing other unwanted DNA fragments. The biotinylated oligos containing di, tri and tetra repeats were hybridized to the DNA fragments that they most closely match at temperature slightly above or below the oligos melting temperature. This product (DNA + probe mix) was added to the washed streptovidin beads. These beads captured the biotinylated probes hybridized to the DNA under magnetic particle collecting (MPC) unit. Again these beads were washed with 2x and 1x hybridization and washing solutions to remove other DNA fragments. At the end 200µl TE (Tris EDTA) buffer was added, vortexed and incubated at 95^{0}C for 5 min, and then captured using MPC unit. The supernatant containing the enriched fragments of microsatellite repeats pool (i.e., "GOLD") was collected. This enriched product was further precipitated with 95% ethanol and resuspended in 25µl of TE buffer (pure "GOLD").

To increase the amount of enriched microsatellite repeat fragments (pure GOLD DNA), PCR was performed using pure gold DNA as template to recover the enriched product and the PCR product was electrophoresed on 1.0% agarose gel.

4.1c Cloning and transformation of plasmid DNA

The amplified enriched recovered microsatellite repeat fragments (pure gold DNA) were cloned into pTZ57R/T vector and transferred into *E. coli*. Based on blue white selection, the presumed recombinant clones were confirmed by colony PCR using the universal M13 primers, and product was resolved on 1.0% agarose gel (Plate 3).

4.1d Screening and sequencing of recombinant clones

More than two thousand colonies were screened by colony PCR and 1021 recombinants with fragments size of 500-1000bp were selected for sequencing. These PCR products were purified using PCR purification kit (SIGMA) and sequenced with nested M13 forward and reverse primers using ABI prism sequencer. The sequence information revealed that most of the sequences varied in size between 500-1000bp

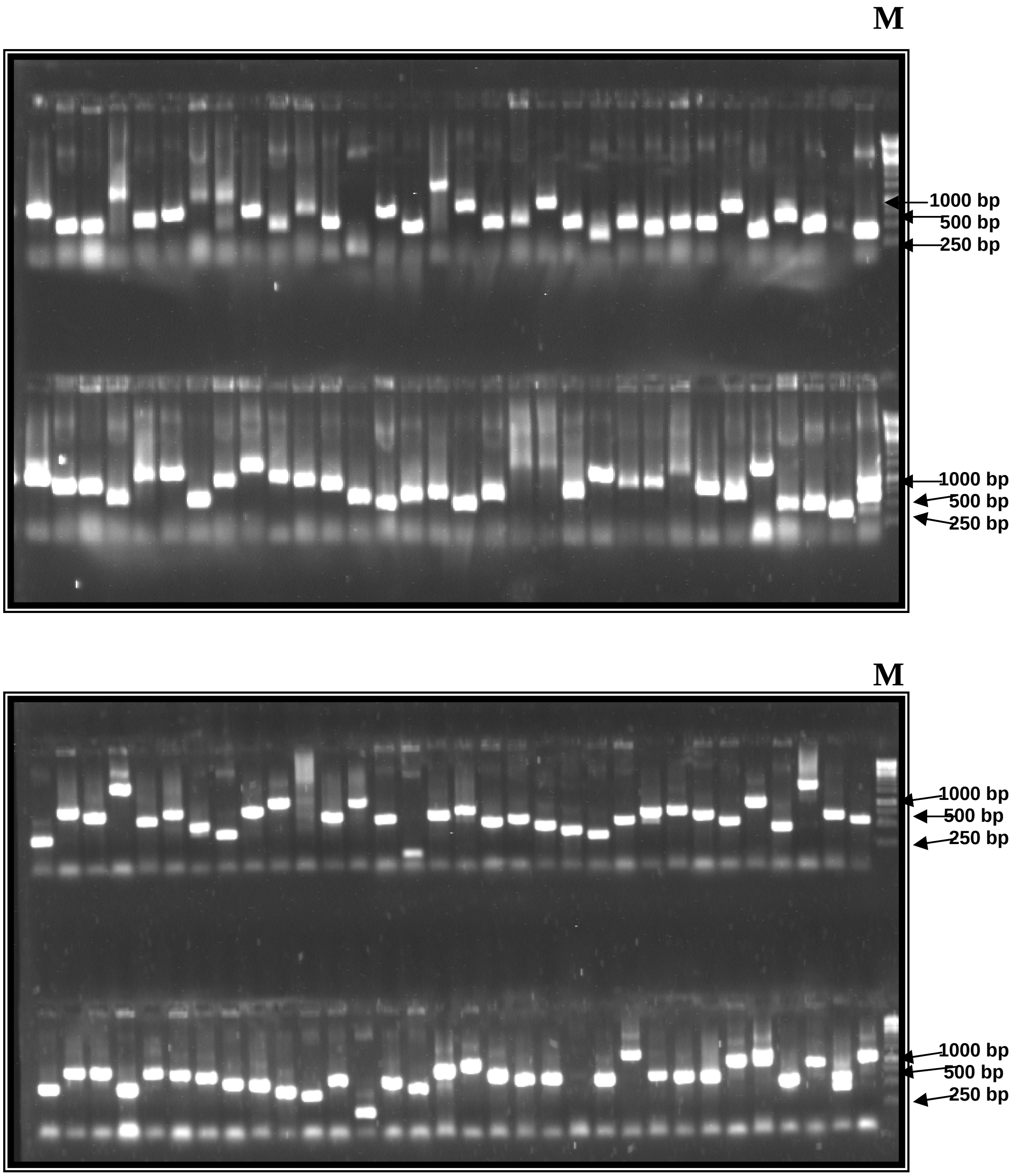


Plate 3 : Screening of the microsatellite enriched colonies by colony PCR

The amplified, enriched, recovered microsatellite repeat fragments (pure gold DNA) were cloned into pTZ57R/T (T/A cloning vector) and transferred into *E.coli*. Based on blue white selection, the recombinant clones were confirmed by colony PCR reactions and product was resolved on 1.0% agarose gel.; M-Marker(1kb)

The sequences were analyzed for the presence of microsatellite repeat regions using the web-based Tandem Repeat finder programme (http://bioinfo.lifl.fr/mreps/mreps.php). Out of the 1021 sequences, 645 sequences contained the microsatellite repeats. The generated sequence information from the library consisted more of di-nucleotide repeats (67%) than mono (11%), tri (14%) tetra (4%) and penta (4%) nucleotide repeats (Table 8). In di-nucleotide repeats, the CT-based repeat motifs were the most abundant (30%) in the library followed by AG repeats (21%) (Table 9). Among tri-nucleotide repeats TGA and TCA were found to be abundant (Table 10). Tetra and penta-nucleotide repeats were relatively less in the enrichment library compared to mono, di and tri-nucleotide repeats (Tables 11&12).The summary of the total number of sequences generated in library is shown in Table 13.

4.1e Primer designing and standardization of annealing temperature

A total of 323 primers were designed to the flanking regions of microsatellite repeats (>15bp) by FastPCR programme. Optimum annealing temperatures were standardized for 323 microsatellite primers by setting up a gradient PCR (Eppendorf) of 55 ± 5^0C and 60 ± 5^0C to obtain specific amplification (Plate 4). About 156 primers showed locus specific amplification. A few primers could not be optimized and they either produced faint bands or did not show reproducible banding patterns. Most of primers generated sharp bands under the programme of 55 ± 5^0C and at 60 ± 5^0C the bands were not sharp and often characterized with a smear or spurious bands presumably due to the high stringent annealing temperature. The details of the novel genomic SSR markers developed in the present study along with the primer sequences, standardized annealing temperature and product length are summarized in Table 14. About 156 locus specific genomic SSR primers have been submitted to NCBI and accession numbers are given in Table 15. Submission of the remaining SSR primers is in progress.

4.2 Development of genic SSRs from stress specific finger millet EST

The existing EST database was used as a source for identifying geneic SSRs. Around 796 stress specific ESTs clones were annotated for the presence of microsatellite repeats using web based Tandem Repeat Finder and primers were designed in the flanking sequence of these repeat regions using FastPCR. In Genic SSR enriched clones, more of monorepeats

Table 8 : Different types of nucleotide repeats and their frequency in enrichment library

Type of Nucleotide repeats	Number of Nucleotide repeat motifs in clones
Monorepeats	165
Direpeats	1047
Trirepeats	207
Tetrarepeats	58
Pentarepeats	62
Total	**1539**

The generated sequence information from the library designated more of di-nucleotide repeats (67%) than mono (11%), tri (14%) tetra (4%) and penta (4%) nucleotide repeats

Table 9 : Distribution and characterization of isolated microsatellite motifs (>15bp) mono & di-repeat groups

Repeat group	Repeat family	Number of repeat motifs in clones
Mononucleotides	A	36
	T	72
	G	18
	C	39
	Total	**165**
Di-nucleotides	AG	336
	AC	6
	TG	9
	TC	111
	GA	96
	CG	3
	GT	6
	CA	3
	CT	474
	TA	3
	Total	**1047**

Enrichment strategy resulted in successful isolation of very good number of di- nucleotide repeats (67%) and few mono nucleotide repeats (11%) abundant in CT and C repeats respectively with repeat length >15 bp

Table 10 : Distribution and characterization of isolated microsatellite motifs (>15bp) in trirepeat groups

Repeat group	**Repeat family**	**Number of repeat motifs in clones**
		6
	TCT	9
	TGA	39
	TGT	6
	GAT	18
	AAG	9
	ATG	6
	GAG	6
	AGG	6
	TGC	3
Trinucleotides	AGA	2
	GTC	1
	GCG	9
	AGC	6
	GAA	12
	TCA	21
	TCG	6
	CAG	6
	CAC	6
	TTG	6
	CTT	9
	ATC	3
	GTT	6
	GAA	6
	Total	207

The second best repeat class in isolation of microsatellites was trirepeats (14%) next to direpeats consisting more of TCA and TGA repeats with > 15 bp repeat length.

Table 11 : Distribution and characterization of isolated microsatellite motifs (>15bp) in tetra-repeat groups

Repeat group	Repeat family	Number of repeat motifs in clones
Tetra nucleotides	AGAC	3
	CACG	3
	TTTC	6
	GCGT	6
	CTCC	3
	GCAT	6
	GCGG	3
	CTGC	6
	CTTC	3
	TTAT	6
	TCCT	1
	CCCT	6
	CGCA	6
	Total	58

The least successful class of repeats in the overall enrichment was tetra nucleotides contributing only 58 repeat motifs in the clones.

Table 12 : Distribution and characterization of isolated microsatellite motifs (>15bp) in penta-repeat groups

Repeat group	Repeat family	Number of repeat motifs in clones
Penta nucleotides	ACTAC	3
	GAAAA	6
	AACAC	3
	AGAGT	3
	TGTGA	3
	ATATT	1
	GCATA	2
	CCCAT	3
	AGCAG	1
	TCTCC	3
	AAACA	3
	CGAGA	3
	TTTGT	3
	TCTTT	4
	CCCCA	3
	CCCCT	3
	CGCCC	3
	CGGCG	3
	CTCCT	3
	CTCCC	2
	GGGGA	1
	AGAGG	3
	Total	62

Among all types of repeats penta nucleotide repeats contribute only to the extent of 4% which is also a less successful group in the enrichment

Table 13 : Summary of sequences generated from the enrichment library

No. of colonies screened	No. of clones sequenced	Clones with SSR motif	Clones without SSR motif	Clones with repeats > 15 bp	No. of primers designed
2200	1021	645	376	323	323

More than 2000 clones were screened to get the bigger fragment size, however one thousand and twenty one bigger fragment clones ranging between 500-1000bp were sequenced with nested M13 forward and reverse primers using ABI prism sequencer. The sequences were analyzed for the repeat motifs regions using the web-based Tandem Repeats finder programme. The results revealed that three hundred and twenty thee repeat motifs were identified with >15bp, for which primers have been designed.

Table 14 : Details of Genomic SSRs developed and standardised in the study

S.N.	Name	Sequence	AT	PS(bp	Remarks
1	FMSSR26FP	ATCTGGCCTTCCGACAGCTTC	60	301	Monomorphic
	FMSSR26RP	CAAGGAGGTCCCCGACGA			
2	FMSSR29FP	CCTCCCTAGCAGAATCAA	60	173	Monomorphic
	FMSSR29RP	GTGTATATTTGTGCCAAATTTTAA			
3	FMSSR35FP	CATAGGACTGAGAAGCTCCCC	60	185	Polymorphic
	FMSSR35RP	AGAGAACAAACAGCTAGACGGA			
4	FMSSR41FP	AATCACCTTTTGCTTCTTTGG	60	119	Monomorphic
	FMSSR41RP	CCCTCTCTAGAGTAAGATTG			
5	FMSSR44AFP	GTTTGGCCTAGCTAGCAG	60	123	Monomorphic
	FMSSR44ARP	GGAAAAGATACTCTCTCTCTC			
6	FMSSR44BFP	TAGAGAGAGAGAGTATCTTTTCC	60	228	Monomorphic
	FMSSR44BRP	TCCGATTGTTTAAGGCCTAG			
7	FMSSR44CFP	AGTCGTATTAGAGCTTGGCGTAC	60	301	Monomorphic
	FMSSR44CRP	ATAACTCCAGGGGTACGTG			
8	FMSSR56FP	TCAAGTAGCTCCTCTTACCTATC	60	160	Monomorphic
	FMSSR56RP	GTAGCTCCTCTTACCTATC			
9	FMSSR77FP	GTAGCTCCTCTTACCTATC	60	248	Monomorphic
	FMSSR77RP	ACCTGCAGTCAGCACTCAG			
10	FMSSR80FP	CCTAGCTGCAGAATCACGG	60	204	Monomorphic
	FMSSR80RP	TGACTGTCAAGTGACGCG			
11	FMSSR83FP	CGGGTAGATACATCTCAGTCG	60	255	Monomorphic
	FMSSR83RP	GTGTCATTAGGGTCCCAAGG			
12	FMSSR84(A)FP	TTTTGCTTGTTCTGATCTTACTCTA	60	160	Monomorphic
	FMSSR84(A)RP	GCTTGTTCTGATCTTACTC			
13	FMSSR84(B)FP	AGAGTAAGATCAGAACAAGC	60	260	Monomorphic
	FMSSR84(B)RP	CTAGCAGAATCACCCTTTG			
14	FMSSR86FP	TGATCATTCTCATTGCTGCTGC	60	153	Monomorphic
	FMSSR86RP	TCCTATGGTAGAGAGGATCCTGC			
15	FMSSR91FP	AGCTGGTGTTTCCTCCACG	60	174	Polymorphic
	FMSSR91RP	CCTTGGCATTGAAGCAACAAG			
16	FMSSR98FP	CGACACATGGAGATGCCG	60	160	Monomorphic
	FMSSR98RP	AGCTAGCAGAATCACTGCA			
17	FMSSR130FP	TCTCGTCACGTCAAGAGC	60	202	Polymorphic
	FMSSR130RP	GACCCCGTGTGGCGTATG			
18	FMSSR145AFP	AATCTCTCTCCCTCTAGAGTAAGATC	60	166	Monomorphic
	FMSSR145ARP	TCTCTCTCCCTCTAGAGTAAGA			

Continued...

19	FMSSR145BFP	GTGAGAGTATATTTGGCTTG	60	297	Monomorphic
	FMSSR145BRP	TAGGAACACACCCACAGA			
20	FMSSR145CFP	GAGCTCTTTGTCACACCCA	60	160	Monomorphic
	FMSSR145CRP	GTCACACCCATAGAGTAGGA			
21	FMSSR153AFP	CCAAGTCACGATTAGTTTG	60	78	Monomorphic
	FMSSR153ARP	CTAGAGTAAGATCAAAACAAGC			
22	FMSSR153BFP	TGCTTATTTCGATCTTACTC	60	160	Monomorphic
	FMSSR153BRP	GAGACACGCAAAAGATAC			
23	FMSSR153CFP	GTATCTTTTGCGTGTCTCTCTCTCAC	60	375	Monomorphic
	FMSSR153CRP	GCGCACGCGTCTGCACTA			
24	SSRFM132FP	5’-GGCCTAGCTAGCAGAATCGC	60	293	Monomorphic
	SSRFM132RP	5'-AGCTAGCAGAATCACCTTTTGC			
25	SSRFM134FP	5’- GCCTAGCTAGCAGAATCACG	60	274	Monomorphic
	SSRFM134RP	5'- CGAGAGAGAGTATCTTTTGC			
26	FMSSR 51F	TCGTGAGTGTGGATGTGTC	55-65	250	Polymorphic
	FMSSR 51R	CCTCAACCTTCTCTGGCATG			
27	FMSSR 61F	CGCAAGGCCCGGACAATG	55-65	205	Polymorphic
	FMSSR 61R	GTGTCTCGTGTGGCCGATC			
28	FMSSR 1A1 F	TCAGCACGGGTTCGCCCTGAG	60	223	Polymorphic
	FMSSR 1A1 R	AGTGCTCGTGCCCCATAGCC			
29	FMSSR 1A2 F	CGTCTGATTGTTTAGGCCTAGC	60	321	Monomorphic
	FMSSR 1A2 R	GCGAGAGAGAGAGAGAGTGCGAC			
30	FMSSR 2A2 F	CGTCTGATTGTTTAGGCCTAGC	60	378	Monomorphic
	FMSSR 2A2 R	GTAACTTTTTCTCGCGTAGCACC			
31	FMSSR 3A6 F	TGTTTTGCGTGTCTCTCCCCGTG	60	245	Monomorphic
	FMSSR 3A6 R	GGAGACTGTGTGAGCTCAACAC			
32	FMSSR 1B2 F	TGCACCATGCTAGACTTCTGCG	60	207	Monomorphic
	FMSSR 1B2 R	CCACAAGAATCTCCGTGCTCTCG			
33	FMSSR 1B6 F	TCGCCATTGGACGGGACTTGG	60	188	Monomorphic
	FMSSR 1B6 R	TATGCAACACCTCCCAGGGGCTG			
34	FMSSR 1B8 F	AGACTTGAGCAAGGCTCCTCCG	60	208	Monomorphic
	FMSSR 1B8 R	TCAGTTGACGGGCCCGGGGTTC			
35	FMSSR 1B12 F	CTGCCGTCAACGATCTGGCC	60	270	Polymorphic
	FMSSR 1B12 R	GCTCGTGCTCTACCGCGATGG			
36	FMSSR 1C1 F	TCGCCATTGGACGGGACTTGG	60	231	Polymorphic
	FMSSR 1C1 R	TCGCCCCAACCCCCGGCTGAAAC			
37	FMSSR 1C5 F	CAGGTTCTCCGGCTTGGGTC	60	208	Monomorphic
	FMSSR 1C5 R	GGTCATTTTGCTAAGGAGTGTCG			
38	FMSSR 1C6 F	AGGAGTAGCAGCAGCCCGCTC	60	298	Monomorphic
	FMSSR 1C6 R	ATGCAGGCCTCTGCAGTCGA			

Continued…

39	FMSSR 1C8 F	CATAGGACTGAGAAGCTCCCCC	60	171	Monomorphic
	FMSSR 1C8 R	CTAGACGGACAAACACACACACG			
40	FMSSR 1C12 F	TAGCAGAATCACCGGTTCACC	60	203	Monomorphic
	FMSSR 1C12 R	GGCATTTCGTGAAGGAATGCAC			
41	FMSSR 1D6 F	TCAGGATCCAAACACCCCATCA	60	175	Polymorphic
	FMSSR 1D6 R	TGTCGTGGTAAGCTTTGCTCC			
42	FMSSR 1D7 F	TGGGAGATCAGACGAAGCAAGC	60	226	Monomorphic
	FMSSR 1D7 R	ACACTACCAATTCTACAGCCCC			
43	FMSSR 2D7 F	AGCGAGATAACGAACAAGCGC	60	165	Monomorphic
	FMSSR 2D7 R	ACACTACCAATTCTACAGCCCC			
44	FMSSR 1D9 F	GTGTGGATGTGTCCGTGTTCG	60	270	Monomorphic
	FMSSR 1D9 R	AGAAACAGGACAATAGGGCCCAC			
45	FMSSR 1E2 F	AGGTTGTCGAATTGGAGACCGC	60	151	Monomorphic
	FMSSR 1E2 R	CCATCAGCCTTATACACCGTTGC			
46	FMSSR 2E2 F	AGGTTGTCGAATTGGAGACCGC	60	196	Monomorphic
	FMSSR 2E2 R	CGACGTCGTAACCTTGCGG			
47	FMSSR 1F2 F	CCTAGTAGCAGAATCACGCCCC	60	173	Monomorphic
	FMSSR 1F2 R	CCCTGGAGTTAGTTACCGGCG			
48	FMSSR 3F2 F	CCGTCAACTGCAAAGGCCTGC	60	278	Monomorphic
	FMSSR 3F2 R	ACACCACCAACCCACACACCAC			
49	FMSSR 1G11 F	TGTCCGGGATGCCTGGGGTAC	60	164	Monomorphic
	FMSSR 1G11 R	CCCTGCTTTTGATCTGACGGAGG			
50	SSRFM3aFP	5'-AGAAGATACTCTGTCTCTGA	50-52	170	Monomorphic
	SSRFM3aRP	5'-CAGAGATAGAAGCTCACGCACG			
51	SSRFM6FP	5'-GCCTAGCTAGCAGAATCACCG	48	119	Monomorphic
	SSRFM6RP	5'-AGATACCCTCCCTCTCTCTAGA			
52	SSRFM12FP	5'-CTCTCTAGTGTGGGCTCAGAA	50	311	Monomorphic
	SSRFM12RP	5'-CGCACCACTACACGTCAGCTG			
53	SSRFM14FP	5'-TCATGTGATGATGAATCTGCAGC	57	250	Monomorphic
	SSRFM14RP	5'-GATGACTTCTTCAATTGGGAGGA			
54	SSRFM16FP	5'-AGCTAGCAGAATCACTGTCCC	57	253	Monomorphic
	SSRFM16RP	5'-CGAGATGTGTGCGTGCGTGTGTA			
55	SSRFM18FP	5'-AGCTAGCAGAATCACCATTTGC	60	449	Monomorphic
	SSRFM18RP	5'-ACGAGATATGTGCGAGGGTGG			
56	SSRFM21bFP	5'-GCTACACTTGCCATTGGCGTCAC	60	544	Monomorphic
	SSRFM21bRP	5'-CTTTGCTAGTGGAAGCATGAGAGG			
57	SSRFM25FP	5'-CCTTCTCTGAATCCGACTCGAA	60	141	Monomorphic
	SSRFM25RP	5'-TGGATCCTCTTGCCATGAAGC			
58	SSRFM28FP	5'-AGCTAGCAGAATCACCTTTTG	50	310	Monomorphic
	SSRFM28RP	5'-GATCCGATTGTTTAAGGCCTAGC			

Continued…

59	SSRFM29FP	5'-ACAAGCAGGATATCCCTTTAGC	60	248	Monomorphic
	SSRFM29RP	5'-ATATAGAGTGTGTCGCCCCCTC			
60	SSRFM34FP	5'-AGCTAGCAGATCACCTTTTGC	60	156	Monomorphic
	SSRFM34RP	5'-CTCTCTCTCGAGTAAGTTCG			
61	SSRFM35aFP	5'-TGGTGATGGTTGAAGGACCAG	59	630	Monomorphic
	SSRFM35aRP	5'-GAAGACGCCTTCTCACCTCTGC			
62	SSRFM35bFP	5'-GAGAGTCTCTTGTGCGTGTGC	60	259	Monomorphic
	SSRFM35bRP	5'-AGCAGCGCCCACACCCAAAGAG			
63	SSRFM44FP	5'-GTGGTGAAGATAAAGCTGCC	60	315	Monomorphic
	SSRFM44RP	5'-GAGTGCACAAACCAACCACA			
64	SSRFM48aFP	5'-ACGTCAACAGCGGCTGGACGA	60	241	Monomorphic
	SSRFM48aRP	5'-GCATTGCTTGGTGTTGGTAGCA			
65	SSRFM48bFP	5'-CATAGGAGATGAGAGCAC	60	91	Monomorphic
	SSRFM48bRP	5'-TCATCATTCTGCTTCATCTTCA			
66	SSRFM58FP	5'-GTCGCCTGTAGCTGAGCAGTC	60	141	Monomorphic
	SSRFM58RP	5'-CCGGTTCGAGAGCTCCGAAGGA			
67	SSRFM66FP	5'-GAATCGCAAGTGTGCAAC	60	236	Monomorphic
	SSRFM66RP	5'-TTTGGTGCTTTGATGCC			
68	SSRFM81FP	5'-GCGACGGTCGAGTAGATG	60	121	Monomorphic
	SSRFM81RP	5'-AGTTCGACACATTGTTGC			
69	SSRFM84FP	5'-AGTAAAAGTGACACGAGCAAGAG	60	356	Monomorphic
	SSRFM84RP	5'-TAGCAGACAGCGCAGCAGCTACTG			
70	SSRFM88FP	5'-CGACTGGCAGGCAGAAGC	60	104	Monomorphic
	SSRFM88RP	5'-GGCATAAAGAAGTGCACGCAC			
71	SSRFM95FP	5'-CTGGTTTGAGGAAGTCCCA	60	192	Monomorphic
	SSRFM95RP	5'-GGAGCTGCATCCACACC			
72	SSRFM100FP	5'-CTCACACCTTAAGACTGCAGC	60	170	Monomorphic
	SSRFM100RP	5'-TCTCTGGAGAGCAGGGCAG			
73	SSRFM101FP	5'-CTCTCTCTCTGGGGAGTGAGCG	52	194	Monomorphic
	SSRFM101RP	5'-GACACTACTGCGCTCTTTGTCG			
74	SSRFM103FP	5'-GGAGGACAGATATCTCAGGC	60	184	Monomorphic
	SSRFM103RP	5'-CCGGATCGATGTTAAGGCTAGC			
75	SSRFM104FP	5'-AAGAACTGGCTACAGGACCC	60	500	Monomorphic
	SSRFM104RP	5'-AACGTCCACTCGGACAGGC			
76	SSRFM109FP	5'-AAGATACCCTAACCCTCAAGC	50	479	Monomorphic
	SSRFM109RP	5'-GGAGCGACGACCAACAC			
77	SSRFM110FP	5'-GAGTGAATGTGCCCATTGG	60	271	Monomorphic
	SSRFM110RP	5'-GGGCAATCGACAATCATGTG			
78	SSRFM115FP	5'-GAGTCGCGCTCGCCTTTGCC	60	167	Monomorphic
	SSRFM115RP	5'-TTCACTGCCGCCGAGGTGACC			

Continued…

79	SSRFM116FP	5'-GTTGCCTGGGTGTTTCC	60	243	Monomorphic
	SSRFM116RP	5'-GACAGATGGAAGAGCAGG			
80	SSRFM119FP	5'-CTCCATGACTGTTGGATGACC	52	110	Monomorphic
	SSRFM119RP	5'-GTCATCACTCTGAGCTACCC			
81	SSRFM120FP	5'-GAAGCGCACTATGATCCCA	60	257	Monomorphic
	SSRFM120RP	5'-ATCTCGCCAAGAGATGCCC			
82	SSRFM129FP	5'-GACCCAAAACCAAACACACCC	60	257	Monomorphic
	SSRFM129RP	5'-GGATCCGATTGTTTAAGGCC			
83	SSRFM135FP	5'-AGGCCTCCCTAGCACAATCAC	60	438	Monomorphic
	SSRFM135RP	5'-AAAACACACTGCTGCTGCTCTGC			
84	SSRFM147FP	5'-GGCGTTACTCCCACCAAC	60	249	Monomorphic
	SSRFM147RP	5'-TGTTTTTCAAGAAGCTGCCTC			
85	SSRFM149FP	5'-TCCGTACAGGCGCCTCCCAG	60	274	Monomorphic
	SSRFM149RP	5'-ACCACAGACTCCCCACCAACC			
86	SSRFM151FP	5'-TTGTTTAGGCCTAGCTAGCAG	52	480	Monomorphic
	SSRFM151RP	5'-TCTAGCTCGCACACTCAGAG			
87	SSRFM153FP	5'-ATCACTTTGCCCGAGAATGCC	60	130	Monomorphic
	SSRFM153RP	5'-CTCTTGTCCTTGCTGAAGTGC			
88	SSRFM156FP	5'-GCAGAATCGCCTAGTTCCC	55	140	Monomorphic
	SSRFM156RP	5'-GGTCAAGCAAGCTAGCAACA			
89	SSRFM157FP	5'-CGCGCGAGAGACACTCTCTG	55	104	Monomorphic
	SSRFM157RP	5'-AGTGTCTCTTACGCACGGGTGC			
90	SSRFM163FP	5'-GTGCCTTCAAGCTCGGGCTCGC	60	133	Monomorphic
	SSRFM163RP	5'-CTTGGATGGGTGACCTCCTGGG			
91	SSRFM170FP	5'-AGAGAGTGAAACCAGCCAGAG	52	128	Monomorphic
	SSRFM170RP	5'-GAGAGAGAGTCTCTTTGCTCGC			
92	SSRFM178FP	5'-GGGATGACCCCATCCCTAGAG	60	177	Monomorphic
	SSRFM178RP	5'-ATGGTGATGGTGGTGGTGACG			
93	SSRFM183FP	5'-CGAACTAGCTCAATACAAGAGCC	60	181	Monomorphic
	SSRFM183RP	5'-GGTGGATCTGGATCTGGATCG			
94	SSRFM186FP	5'-AAAATCCCCAGAACCAACCTCG	60	194	Monomorphic
	SSRFM186RP	5'-GAAGATGAGTAGGCGAGGAGG			
95	SSRFM190FP	5'-AGCTAGCAGAATCACTCCC	56	436	Monomorphic
	SSRFM190RP	5'-AGACCTAGCTAGCACAATCTCCC			
96	SSRFM196FP	5'-CCTCCAAATCCGACTTGG	60	145	Monomorphic
	SSRFM196RP	5'-GATACCTTGGATCCTCTTGCC			
97	SSRFM201FP	5'-TGGTAGCAATTGAATGGCGCG	60	179	Monomorphic
	SSRFM201RP	5'-GCATTTGGGCCCGTTGGAGC			
98	SSRFM203FP	5'-GCTAGTAGCAGAATCACTCTCTCG	58	216	Monomorphic
	SSRFM203RP	5'-AGCTAGCAGAATCGCCTTTTGC			

Continued…

99	SSRFM204FP	5'-CTTTCACCGACCGCGTGTCG	60	110	Monomorphic
	SSRFM204RP	5'-GAAACCCGACGTCGCATCCC			
100	SSRFM219FP	5'-GTGCAAGCAAAGTGGTTGAGG	60	154	Monomorphic
	SSRFM219RP	5'-TCTGTAGTGGTGGAAGCAGC			
101	SSRFM226FP	5'-GGTGTGAGGATCTACCTTGC	60	173	Monomorphic
	SSRFM226RP	5'-GCTAGCAGAATCACATCTCCG			
102	SSRFM236FP	5'-TGCAAGCAAAGTGGTTGAGGC	60	153	Monomorphic
	SSRFM236RP	5'-TCTGTAGTGGTGGAAGCAGCAG			
103	SSRFM243FP	5'-CTATGGATAGTGTCCACCATGC	60	240	Monomorphic
	SSRFM243RP	5'-CGAAGCATCATGGGGCTTGG			
104	SSRFM252FP	5'-ACTCTCCAACAAGTGCCTTGC	60	217	Monomorphic
	SSRFM252RP	5'-AGGCAAGTGCTTCAATTGTGGC			
105	SSRFM254FP	5'-TATTCATGGAGCCGCCAACGG	60		Monomorphic
	SSRFM254RP	5'-GCGCTATGCTTATGTCGTAGG			
106	SSRFM255FP	5'-GAGGATGTCACCTCAGCGGC	60	200	Monomorphic
	SSRFM255RP	5'-AGGCGCAAGACGACGGTGAAG			
107	SSRFM256FP	5'-TGTGCAAGCAAAGTGGTTGAGG	60	157	Monomorphic
	SSRFM256RP	5'-CTTCTGTAGTGGTGGAAGCAGC			
108	SSRFM257FP	5'-TTGGTTTTTGGGGGGTTTAGG	50	326	Monomorphic
	SSRFM257RP	5'-CAATACAAAGCCCAATGCGAC			
109	SSRFM272FP	5'-CTAGCTAGCAGAATCCCATACAC	53	321	Monomorphic
	SSRFM272RP	5'-GCTTGTTTCGATCACACTCACG			
110	SSRFM273FP	5'-CCGGACCAAGCATAAGTCACGC	60	495	Monomorphic
	SSRFM273RP	5'-TGGAGTATTGTGCGCATGTGC			
111	SSRFM275FP	5'-TGCCACTTCTGCGTCACGCC	60	309	Monomorphic
	SSRFM275RP	5'-GAGAAGGCCATTGGGGGCTACG			
112	SSRFM282FP	5'-TTCAGAGCAAACCCATCGTGG	60	211	Monomorphic
	SSRFM282RP	5'-ATGCCCTGTGTGCCCTCG			
113	SSRFM283FP	5'-GAGAGAGGGAGTATCTTTTGCG	52	189	Monomorphic
	SSRFM283RP	5'-TATATCATGGCGCGAGTCAC			
114	SSRFM284FP	5'-TTTAGGCCTAGCTAGCAG	50	368	Monomorphic
	SSRFM284RP	5'-AAAGATACTGTCTCGCACTCAC			
115	SSRFM285FP	5'-ACACTCCGTGTGTGGCCC	52	445	Monomorphic
	SSRFM285RP	5'-AGCTAGCAGAATCACGGCTCC			
116	SSRFM286FP	5'-AACAGGCAAACCGGGCAAAGC	59	246	Monomorphic
	SSRFM286RP	5'-GCCCTCCTGCTCGCTGC			
117	SSRFM290FP	5'-GAGTCATTTTGATCTATGATCTC	59	362	Monomorphic
	SSRFM290RP	5'-CAACATGCTAGCTACGAACGA			
118	SSRFM295FP	5'-GCCCGGTCGCAGAATCACC	59	148	Monomorphic
	SSRFM295RP	5'-TTCCAAGTCAAGGGGCAAGTGC			

Continued…

119	SSRFM296FP	5'-GCAAGGTGACCTCAAGACCAAG	60	266	Monomorphic
	SSRFM296RP	5'-AGTTGAAGCACTTGCCCTTGG			
120	SSRFM299FP	5'-CTGGATTGGCTCAGCATG	52	347	Monomorphic
	SSRFM299RP	5'-CTTGAACTTGGAAGCGGATAC			
121	SSRFM10FP	5'-GCTAGCAGAATCACCTTTTGC	57	461	Monomorphic
	SSRFM10RP	5'-GATTGTTTAAGGCCTAGTGCAG			
122	SSRFM301FP	5'-GCCATCTGCAGAATCACGACTG	51	228	Monomorphic
	SSRFM301RP	5'-ACCTGTATCGCTCTCACACTACG			
123	SSRFM304FP	5'-GCCTAGCTAGCAGAATCACCC	55	156	Monomorphic
	SSRFM304RP	5'-AGTGTCACCCTCTCGTCTGGC			
124	SSRFM343FP	5'-AGTGGAGCATCCAAGATCAAGG	60	219	Monomorphic
	SSRFM343RP	5'-CTGGGTCATGGCTCATGGAAG			
125	SSRFM352FP	5'-AAGCTTGTTTCCTGAGACCCC	55	374	Monomorphic
	SSRFM352RP	5'-GAGGATGATGTTGGGGGG			
126	SSRFM353FP	5'-GTGTCTGTGCCCGCGTATGTG	55-60	339	Monomorphic
	SSRFM353RP	5'-GGTGTGGTCAGAAGCACACAC			
127	SSRFM354FP	5'-CCCCTGCAGAATCACCCAGAC	55-60	166	Monomorphic
	SSRFM354RP	5'-CTGATGAGGAGCTGTCGTTGC			
128	SSRFM355FP	5'-TTCTTGGGCTTGGGGCAATCC	55-60	247	Monomorphic
	SSRFM355RP	5'-GGATGATCCTCAACCATCCAACC			
129	SSRFM359FP	5'-AATCCGAAGCCTGCTGCAAAGG	55-60	167	Monomorphic
	SSRFM359RP	5'-AGCGTTATGCCACTGGCCAGCTC			
130	SSRFM360AFP	5'-TTCTGGAGCGGCGGCAGGAG	62-65	238	Monomorphic
	SSRFM360ARP	5'-TCCAATGGCTCCGCCTCCACC			
131	SSRFM360BFP	5'-TGGTCCTCAGGAAAGAGGGG	55-60	190	Monomorphic
	SSRFM360BRP	5'-TGAATCCCACTCGTCGGGAAC			
132	SSRFM367FP	5'-GCAATCAATCTGGACGTCGCC	55-60	219	Monomorphic
	SSRFM367RP	5'-TCGTGTTTGTTTTCACCCGG			
133	SSRFM393AFP	5'-GTTAAGGCCTACCCGCAG	45-50	139	Monomorphic
	SSRFM393ARP	5'-GAGAGTATCTTTGCCTGTTTCG			
134	SSRFM396AFP	5'-AGCACCAGGATCGCATCTG	50	196	Monomorphic
	SSRFM396ARP	5'-ATTGTTTAAGGCCTCAGCTAGC			
135	SSRFM396BFP	5'-ATAGAGAGAGAGCCACACGTGTC	50	208	Monomorphic
	SSRFM396BRP	5'-CCGCGCACATATCTCTCGCG			
136	SSRFM398AFP	5'-GGATGATCCACAACCATCCG	55-60	199	Monomorphic
	SSRFM398ARP	5'-CAGTTGAAGCACTTGCCCTTGG			
137	SSRFM398BFP	5'-TTCCAAGGGCAAGTGCTTCAA	45-50	167	Monomorphic
	SSRFM398BRP	5'-CTAGCTAGCAGAATCGCATC			
138	SSRFM402FP	5'-TTGTTTAGGCTAGCCAGCAGG	57	220	Monomorphic
	SSRFM402RP	5'-TGGCTTGCAGAGTCGGAGAG			

Continued…

139	SSRFM408FP	5'-TGCAGCAGCTAATAACAGCC	60	251	Monomorphic
	SSRFM408RP	5'-GGGAGAGATCATGGCGAGG			
140	SSRFM409AFP	5'-TAGGCCTACCTAGCAGAATCAC	55	314	Monomorphic
	SSRFM409ARP	5'-CTCTAGAGTGAGGGCAGAGC			
141	SSRFM137FP	5'-TAGGCTAGTAGCAGAATCCACACC	55	313	Monomorphic
	SSRFM137RP	5'-AAAGCTTGCATGCAGGCCTC			
142	SSRFM142FP	5'-CTTTTGAAGGGCCTTTCGAGC	60	267	Monomorphic
	SSRFM142RP	5'-AATACCAGGCACTTCCCGTTCC			
143	SSRFM180FP	5’-GTTTAGGCCTAGCTAGCAG	55-60	259	Monomorphic
	SSRFM180RP	5'-CAGAGTGGCTTTTGCTTG			
144	SSRFM182FP	5’-AGAGAGAGAGTGAAACAAGC	57	200	Monomorphic
	SSRFM182RP	5'-AGCTAGCACAATCACGCATC			
145	SSRFM415 FP	5'-AGATCCTTGCTTCTGAAGCCAA	55-65	200	Monomorphic
	SSRFM415 RP	5'-GTTAATTCATCGCATCGGTGCCG			
146	SSRFM435FP	5'-GAGTGAATGTGCCCATTGGTG	55-65	209	Monomorphic
	SSRFM435RP	5'-ACCCTTGGCCTTGTCAAAGCTC			
147	SSRFM438FP	5'-GTCCTATCAGTGGTGGCAAGC	55-65	274	Monomorphic
	SSRFM438RP	5'-CTTGCGAGGTCTGTGGCAACAC			
148	SSRFM450FP	5'-CAGAATCACCATTGATTAGGGC	55-65	149	Monomorphic
	SSRFM450RP	5'-GAAGACCCCAATCTGCATTTGC			
149	SSRFM454FP	5'-AAATGCTAAGGGGCTCGTTGC	55-65	212	Monomorphic
	SSRFM454RP	5'-GTCTTGCTGGTGGTTGGCATCG			
150	SSRFM455FP	5'-GGTCGTCGAGAACTAAGGAGC	55-65	140	Monomorphic
	SSRFM455RP	5'-GGGAGCTAACCTTTGGGGTCC			
151	SSRFM463FP	5'-ATTGTGCTTGCCCCCTAGCCA	55-65	113	Monomorphic
	SSRFM463RP	5'-TCGGTGTGAGGAGCTACCTTGC			
152	SSRFM465FP	5'- GCAAATCAACCTGAACGCGAG	55-65	171	Monomorphic
	SSRFM465RP	5'-GAGATCTTCGGGCTCTTCTGG			
153	SSRFM467FP	5'-TGGGTGACCTCCTGGGAAGTC	55-65	290	Monomorphic
	SSRFM467RP	5'-ATGCCCGTTCTGATGGGGTCC			
154	SSRFM468FP	5'-AAATGCTAAGGGGCTCGTTGC	55-65	213	Monomorphic
	SSRFM468RP	5'-TGTCTTGCTGGTGGTTGGCA			
155	SSRFM481FP	5'-GGGAGGAGACTATTCCAAGACTC	55-65	210	Monomorphic
	SSRFM481RP	5'-AACTCGTGCACATGGAGCTCTC			
156	SSRFM484FP	5'-CGAAGCCACACCATAGCCAACC	55-65	157	Monomorphic
	SSRFM484RP	5'-CTGCTGTTGTTCGTCCATGG			

Table 15 : The list of GeneBank accession number for finger millet genomic SSRs

Sl No.	dbSTS_Id	User_Id	GenBank_Accn
1	854991	FMSSR35	BV723931
2	854992	FMSSR86	BV723932
3	854993	FMSSR91	BV723933
4	854994	FMSSR130	BV723934
5	854995	FMSSR1A1	BV723935
6	854996	FMSSR1A2	BV723936
7	854997	FMSSR2A2	BV723937
8	854998	FMSSR3A6	BV723938
9	854999	FMSSR1B2	BV723939
10	855000	FMSSR1B6	BV723940
11	855001	FMSSR1B8	BV723941
12	855002	FMSSR1C1	BV723942
13	855003	FMSSR1C5	BV723943
14	855004	FMSSR1C6	BV723944
15	855005	FMSSR1C8	BV723945
16	855006	FMSSR1C12	BV723946
17	855007	FMSSR1D6	BV723947
18	855008	FMSSR1D7	BV723948
19	855009	FMSSR2D7	BV723949
20	855010	FMSSR1D9	BV723950
21	855011	FMSSR1E2	BV723951
22	855012	FMSSR2E2	BV723952
23	855013	FMSSR1F2	BV723953
24	855014	FMSSR3F2	BV723954
25	855015	FMSSR1G11	BV723955
26	855016	FMSSR51	BV723956
27	855017	FMSSR61	BV723957
28	855018	FMSSR1B12	BV723958
29	1238735	SSRFM14	GF107652
30	1238736	SSRFM16	GF107653
31	1238737	SSRFM18	GF107654
32	1238738	SSRFM21b	GF107655
33	1238739	SSRFM25	GF107656
34	1238740	SSRFM29	GF107657
35	1238741	SSRFM34	GF107658
36	1238742	SSRFM35B	GF107659

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37	1238743	SSRFM44	GF107660
38	1238744	SSRFM48a	GF107661
39	1238745	SSRFM48B	GF107662
40	1238746	SSRFM58	GF107663
41	1238747	SSRFM66	GF107664
42	1238748	SSRFM81	GF107665
43	1238749	SSRFM84	GF107666
44	1238750	SSRFM103	GF107667
45	1238751	SSRFM104	GF107668
46	1238752	SSRFM110	GF107669
47	1238753	SSRFM115	GF107670
48	1238754	SSRFM116	GF107671
49	1238755	SSRFM120	GF107672
50	1238756	SSRFM129	GF107673
51	1238757	SSRFM135	GF107674
52	1238758	SSRFM147	GF107675
53	1238759	SSRFM149	GF107676
54	1238760	SSRFM153	GF107677
55	1238761	SSRFM163	GF107678
56	1238762	SSRFM178	GF107679
57	1238763	SSRFM183	GF107680
58	1238764	SSRFM186	GF107681
59	1238765	SSRFM196	GF107682
60	1238766	SSRFM201	GF107683
61	1238767	SSRFM204	GF107684
62	1238768	SSRFM219	GF107685
63	1238769	SSRFM226	GF107686
64	1238770	SSRFM236	GF107687
65	1238771	SSRFM243	GF107688
66	1238772	SSRFM252	GF107689
67	1238773	SSRFM254	GF107690
68	1238774	SSRFM255	GF107691
69	1238775	SSRFM256	GF107692
70	1238776	SSRFM273	GF107693
71	1238777	SSRFM275	GF107694
72	1238778	SSRFM282	GF107695

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73	1238779	SSRFM286	GF107696
74	1238780	SSRFM290	GF107697
75	1238781	SSRFM295	GF107698
76	1238782	SSRFM296	GF107699
77	1238783	SSRFM299	GF107700
78	1238784	SSRFM435	GF107701
79	1238785	SSRFM438	GF107702
80	1238786	SSRFM450	GF107703
81	1238787	SSRFM454	GF107704
82	1238788	SSRFM455	GF107705
83	1238789	SSRFM463	GF107706
84	1238790	SSRFM465	GF107707
85	1238791	SSRFM467	GF107708
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87	1238793	SSRFM481	GF107710
88	1238794	SSRFM484	GF107711
89	1238795	SSRFM3a	GF107712
90	1238796	SSRFM6	GF107713
91	1238797	SSRFM12	GF107714
92	1238798	SSRFM28	GF107715
93	1238799	SSRFM35a	GF107716
94	1238800	SSRFM109	GF107717
95	1238801	SSRFM119	GF107718
96	1238802	SSRFM151	GF107719
97	1238803	SSRFM156	GF107720
98	1238804	SSRFM157	GF107721
99	1238805	SSRFM170	GF107722
100	1238806	SSRFM190	GF107723
101	1238807	SSRFM203	GF107724
102	1238808	SSRFM257	GF107725
103	1238809	SSRFM272	GF107726
104	1238810	SSRFM283	GF107727
105	1238811	SSRFM284	GF107728
106	1238812	SSRFM285	GF107729
107	1238813	SSRFM301	GF107730
108	1238814	SSRFM304	GF107731

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109	1238815	SSRFM343	GF107732
110	1238816	SSRFM352	GF107733
111	1238817	SSRFM353	GF107734
112	1238818	SSRFM354	GF107735
113	1238819	SSRFM355	GF107736
114	1238820	SSRFM359	GF107737
115	1238821	SSRFM360A	GF107738
116	1238822	SSRFM360B	GF107739
117	1238823	SSRFM367	GF107740
118	1238824	SSRFM393A	GF107741
119	1238825	SSRFM396A	GF107742
120	1238826	SSRFM396B	GF107743
121	1238827	SSRFM398A	GF107744
122	1238828	SSRFM398B	GF107745
123	1238829	SSRFM402	GF107746
124	1238830	SSRFM408	GF107747
125	1238831	SSRFM409A	GF107748
126	1238832	SSRFM132	GF107749
127	1238833	SSRFM134	GF107750
128	1238834	SSRFM137	GF107751
129	1238835	SSRFM180	GF107752
130	1238836	FMSSR26	GF107753
131	1238837	FMSSR29	GF107754
132	1238838	FMSSR41	GF107755
133	1238839	FMSSR44A	GF107756
134	1238840	FMSSR44B	GF107757
135	1238841	FMSSR44C	GF107758
136	1238842	FMSSR77	GF107759
137	1238843	FMSSR80	GF107760
138	1238844	FMSSR84B	GF107761
139	1238845	FMSSR145B	GF107762
140	1238846	FMSSR153A	GF107763
141	1238847	FMSSR153C	GF107764
142	1238848	SSRFM142	GF107765
143	1238849	SSRFM88	GF107766
144	1238850	SSRFM95	GF107767

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145	1238851	SSRFM100	GF107768
146	1238852	SSRFM415	GF107769
147	1238853	SSRFM101	GF107770
148	1238854	SSRFM10	GF107771
149	1238855	SSRFM182	GF107772
150	1238856	FMSSR83	GF107773
151	1238857	FMSSR153B	GF107774
152	1238858	FMSSR56	GF107775
153	1238859	FMSSR84A	GF107776
154	1238860	FMSSR98	GF107777
155	1238861	FMSSR145A	GF107778
156	1238862	FMSSR145C	GF107779

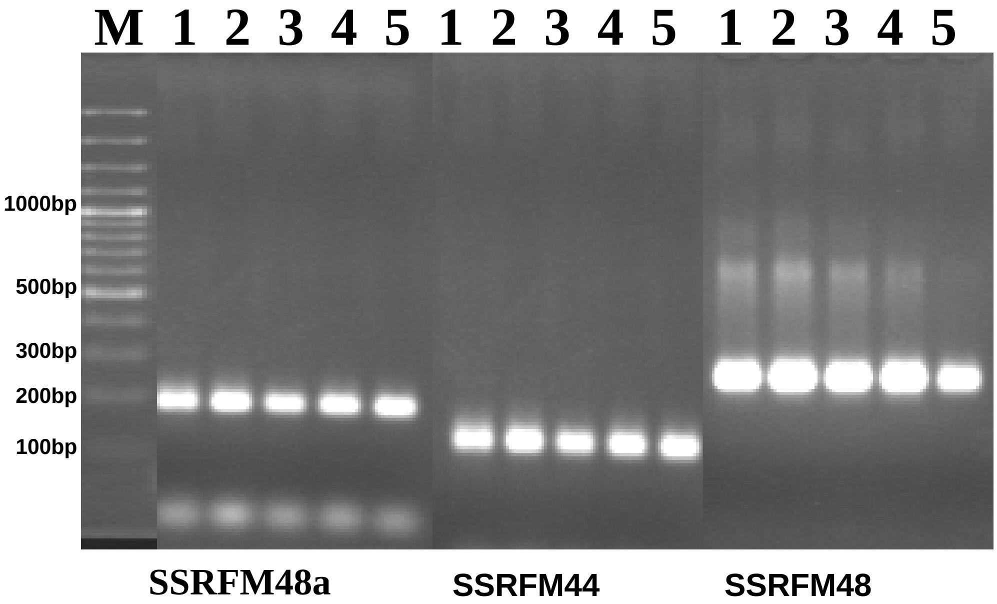


Plate 4 : Standardization of annealing temperature of SSR primers for locus specific amplification

The in-house developed microsatellite primers were initially standardized for optimum annealing temperature at different gradient temperatures. The gel depicts standardization of annealing temperature of few SSR primers Lane 1-59.9^{0}C, 2-61.6^{0}C, 3-64.0^{0}C, 4-66.1^{0}C, 5-69.6^{0}C; M-Marker (100bp)

(187) found followed by direpeats (125), very few tri, tetra and penta repeats were found (Table 16). Around 149 genic SSR primers were designed and synthesized for further validation. These primers were standardized for locus specific amplification by setting a gradient PCR of 60 ± 5^0C. Around 92 primers showed locus specific amplification. The locus specific amplification of a few genic SSRs is depicted in Plate5. The details of genic SSR markers with the primer sequences, standardized annealing temperature and product length are summarized in Table 17

4.3 Utilization of published SSRs of Fingermillet and Rice markers

Rice and finger millet SSR markers available in public domain were used for assessing molecular diversity among finger millet population. In all 31 finger millet SSRs and 228 rice SSRs were selected to assess polymorphism. Plate 6 depicts the standardization of outsourced fingermillet SSR markers. Around 69 rice SSR primers showed locus specific amplification (Plate 7). The details of the outsourced i.e., finger millet and rice markers with the primer sequences, standardized annealing temperature and product length are summarized in Table 18 & 19 respectively.

Physiological characterization

The major emphasis of this study was to generate genomic resources and exploit them in molecular characterization of mapping population leading finally to identify QTLs governing the variability in drought tolerance traits. Towards this end, recombinant inbred population of 150 lines was characterized for phenotypic and molecular diversity.

I Experiment

4.4 Phenotypic evaluation of parents

Based on some previous studies, two *Elusine coracana* lines IE 2912 and IE2885 were identified as contrasts for drought tolerance and neck blast resistance. The parent IE 2912 was resistant for neck blast and drought whereas IE 2885 was susceptible for both these stresses.

Table 16 : Different types of nucleotide repeats and their frequency in Genic SSR enriched clones enrichment library

Type of nucleotide repeat	Number of nucleotide repeats
Mono repeats	187
Di repeats	125
Tri repeats	35
Tetra repeats	19
Penta repeats	25

Around 796 stress specific ESTs clones were annotated for the presence of microsatellite repeats using web based Tandem Repeat Finder. Genic SSR enriched clones, more of monorepeats (187) found followed by direpeats (125), very few tri,tetra and penta repeats were found

Table 17 : Details of Genic/EST SSRs developed in the study

S. N.	Name	Sequence	AT	PS(bp)	Remarks
1	GFMSSR1F_A07	AGACGCAATGAGAAGAGTGGACG	61.5	216	Monomorphic
	GFMSSR1F_A07	ACGCTACTCTACGCTCGCTCAC			
2	GFMSSR1F_C02	GGGATGGGTAGGGAGCGTGC	59.6	240	Monomorphic
	GFMSSR1F_C02	TCGCACTCTCTCTGCTCTCGCAG			
3	GFMSSR1F_C03	GAACGGCCCGGATTGCGACACTG	59-64	196	Monomorphic
	GFMSSR1F_C03	TTTCTCCCCCGCGCTCCCAGAGG			
4	GFMSSR1F_C04	AGACAGAGCGCGGCGAGAAG	59-64	234	Monomorphic
	GFMSSR1F_C04	TCGCGTCTCTCGCTCTCGGCTG			
5	GFMSSR1R_E02	GGATGGGGAGGGGGGTTAG	61.3	225	Monomorphic
	GFMSSR1R_E02	ACCCCCCCTAAGCGCACTCTC			
6	GFMSSR1A08 F(	TGGGGAAAATCGGGGTAGCC	60.4	380	Monomorphic
	GFMSSR1A08 R(	TCTGTCACTCTCGCAGGC			
7	GFMSSR2A08F(1	TGAGCAAGAGATAAGCCTGCG	61.8	114	Monomorphic
	GFMSSR2A08R(	TGACTCTCTCTCACACGGCGAG			
8	GFMSSR1B03F(1	GGCGAAGAGAGAGAGACAGGC	59.1	130	Monomorphic
	GFMSSR1B03R(1	CTCTTTGTGTTTGCCCGCTTG			
9	GFMSSR1E05 F(	GGGGGGATGATGACCGGGAG	59.1	110	Monomorphic
	GFMSSR1E05 R(	TCCTCATTGGCGCGCCTCCGTCG			
10	GFMSSR1E12F(1	CACAGACAAGAGACGAAAGCCCA	64.2	283	Monomorphic
	GFMSSR1E12R(1	TGTTGTTCGTCGTGTTGCTCTGC			
11	GFMSSR1G08F(1	ACGAGAGAGAAAAGGCGGGGA	64.2	270	Monomorphic
	GFMSSR1G08R(	GCGCTCTCTTGCTGCATGGTG			
12	GFMSSR1A07 F(	TGGCAATTACGGCCCGGGCATG	64.2	161	Monomorphic
	GFMSSR1A07R (	TGGGGGGGTTTTGGGGGTTTG			
13	GFMSSR1A09 F(	TGACCTTTCGGCTTCTCCGTGG	69.2	199	Monomorphic
	GFMSSR1A09 R(	ACAAGCAGAGACAAGAGACGAGC			
14	GFMSSR1B02 F(	AGCTGCGTTTTGTGGTGTCTCC	63.1	179	Monomorphic
	GFMSSR1B02 R(	CATCAGGACACGCCGCCGCAGAG			
15	GFMSSR1B08F (	GTACGGCCGGGGGGCATTG	63.1	175	Monomorphic
	GFMSSR1B08R (	CGGGATCCGATTAGATGCTGACG			
16	GFMSSR1H01F (	AGAATGGCTGAACGAGTCGAG	63.1	549	Monomorphic
	GFMSSR1H01R (	TGTTGTTGGTGTTGGGTTGG			
17	GFMSSR1H06F (	AGCCGGAGAAAAACAGGGCGC	64.2	514	Monomorphic
	GFMSSR1H06R (	TGGTCGCTCGTGTGTCGTCTGG			
18	GFMSSR1H12F (	AAAAGCCCCAGGAGACGG	63.1	147	Monomorphic
	GFMSSR1H12R (	TCTCTTCCCTTTCCCCCTCC			

Continued..

19	GFMSSR1 A07F (	AGTAGAGAGACAGAGACGAGC	64.2	221	Monomorphic
	GFMSSR1 A07R	CATACTGCTGCATGCTTCTGC			
20	GFMSSR1 B04F (	ATTTTCCCCAAAAGGGGGCC	64.2	235	Monomorphic
	GFMSSR1 B04R	TCTCTCTCCCTCTCTCCCTCC			
21	GFMSSR1 B07F(	AGAGAGAACACGAGCGAGAG	65-67	135	Monomorphic
	GFMSSR1 B07R(	GTGGTGTGTTTCTTGCTGTCC			
22	GFMSSR2 B07F(	GAACTACCGTGTACGCAGACAG	65.6	155	Monomorphic
	GFMSSR2 B07R(	TGCTGTGTCCTTACTTGTCGTGC			
23	GFMSSR1 B11F (	GGGAAAGAGAGAGCGAGG	65.5	157	Monomorphic
	GFMSSR1 B11R	*TCTCTCTGCTCTGCTGCGG*			
24	GFMSSR1 C08F (	CAGGAGACGGGGAGAGTGCC	69.2	218	Monomorphic
	GFMSSR1 C08R	TCGTCTCGCTCTCGTGGTCGCTG			
25	GFMSSR2D07F (	AGGGAGAGACGAAGACCACGAGG	66.4	132	Monomorphic
	GFMSSR2D07R (	TCGGTCCTCTGCCCGCTCTCGGA			
26	GFMSSR1D11F (	GCCAGAAAAAGAAGAGCCAGAGC	70.5	107	Monomorphic
	GFMSSR1D11R (	TCTGTCTCGGTTGGCGCGATC			
27	GFMSSR1E02F (	CAGCAGAAACAGAGCAAGCAGAC	65.5	138	Monomorphic
	GFMSSR1E02R (	TCGTCGTTGCTGCCTCCTGCA			
28	GFMSSR2 E04F (	ACACAGACACACACACAGGGAGG	65	176	Monomorphic
	GFMSSR2 E04R (	TCTGGTCTGTCTGCTAGCGTGG			
29	GFMSSR1 E09F (	GATCTTCCCCATAAAGCGGCC	60.5	170	Monomorphic
	GFMSSR1 E09R (	TGCATGCAATCGTCGGGTAGTG			
30	GFMSSR2 E09 F(	CATACCACACTACCCGACGATTG	65	140	Monomorphic
	GFMSSR2 E09 R(	TGCTGGTTGATGTGGGGTG			
31	GFMSSR1 G12F (	AAAAGCGGAGAAAACCCCACG	60.5	178	Monomorphic
	GFMSSR1 G12R	TCTCTCTCTCTGCGTGCTTGC			
32	GFMSSR1 H3F (	AACCTGGGGGAAACCCGGC	65	309	Monomorphic
	GFMSSR1 H3R (	TCTCTCTCTTGGTGGCCTGTGG			
33	GFMSSR1 H08 F(	TGTGTTCCGTCTGGCCACTC	65	116	Monomorphic
	GFMSSR1 H08 R	ACCACTTCGCTCCGCGCTCTAC			
34	GFMSSR2 H08 F(	TGTGTGTGTAGTAGAGCGCGGAG	65	204	Monomorphic
	GFMSSR2 H08 F(	ACACACCCTAAAACAGCGCACG			
35	GFMSSR1H03F (	GTGTTGATCTTTTCGTGGGCTCC	68.1	514	Monomorphic
	GFMSSR1H03R (	ACACAAAACAGCACACCACCAC			
36	SSRGFM1FP	5'-GGGTTCCGTACCAACCCC	55-65	191	Monomorphic
	SSRGFM1RP	5'-GGGATTATTTGGCGACGGG			
37	SSRGFM2FP	5'-CGCGCGTGCGTGGTCAGTAG	55-65	176	Monomorphic
	SSRGFM2RP	5'-ACGTCGACTCGCCGACAG			
38	SSRGFM3FP	5'-TAACAACATGTTCCATGCAGC	55-65	238	Monomorphic
	SSRGFM3RP	5'-GAAAGAAGAAGAGAAGAAGAAGAG			

Continued..

39	SSRGFM4FP	5'-GCCGTCCTCCTGCAGCTTGTAGG	55-65	258	Monomorphic
	SSRGFM4RP	5'-CTAGTGGATCCCCCGGGCTGCAGG			
40	SSRGFM5FP	5'-GGCCTCGGTTTGCTGGGG	55-65	146	Monomorphic
	SSRGFM5RP	5'-GCTGCAGTGCCTGCTGCATCAG			
41	SSRGFM6FP	5'-GTGTGTGTATGCGCTGCGTGTG	55-65	159	Monomorphic
	SSRGFM6RP	5'-CGCGCAATCACACAGTACACGT			
42	SSRGFM8FP	5'-AAGGCCTCCAGCATCCCCG	55-65	245	Monomorphic
	SSRGFM8RP	5'-GTGGCAGTGTACACGAGGGAG			
43	SSRGFM9FP	5'-GTTCCACGAGCACCACGACAA	55-65	200	Monomorphic
	SSRGFM9RP	5'-GCACGAAACCCTGGCCTGG			
44	SSRGFM10FP	5'-GATGAGCTCAAGCCCTTCGG	55-65	151	Monomorphic
	SSRGFM10RP	5'-GTTGAGGTGGTGAGTAGGGG			
45	SSRGFM16FP	5'-TCCCATCCTTTTAGCTGCTG	55-65	199	Monomorphic
	SSRGFM16RP	5'-AACACCTTTGCTGCACTCC			
46	SSRGFM24FP	5'-ACGGCCTAATTCCATTCC	60.6	214	Monomorphic
	SSRGFM24RP	5'-AGCCACAAGCCCAACAATTA			
47	SSRGFM25FP	5'-GACAGGCACAGATATGGTGAGA	55.4	228	Monomorphic
	SSRGFM25RP	*5'-TCCTTGGTATGCGTTTTCC*			
48	GFM7FP	5'-CGCGCGTGCGTGGTCAGTAG	55-65	227	Monomorphic
	GFM7RP	5'-AAGAACCGCTCCACCACTCGC			
49	GFM58aFP	5'-GCTGGAGCGATCGCAGG	55-65	178	Monomorphic
	GFM58aRP	5'-CAAGGGAACTCGACAGTCAC			
50	GFM67FP	5'-AGCGCCTCCATCGACTGC	55-65	248	Monomorphic
	GFM67RP	5'-GCTCTCTCGCACGAACACA			
51	GFM69FP	5'-GTATATGCTCTCCATCTACCGG	50-60	211	Monomorphic
	GFM69RP	5'-TCCAGCAGCAAAGCTCGA			
52	GFM76FP	5'-GAGGAATTCTTCGACGCGC	50-60	315	Monomorphic
	GFM76RP	5'-AACAAAACACCAGCCCG			
53	GFM81FP	ATATCTCACCGTGCACTGGG	50-60	224	Monomorphic
	GFM81RP	AGACACACTTCCCAGACAACC			
54	GFM83FP	5'-AATCACACAGTAATAAACAGC	50-60	103	Monomorphic
	GFM83RP	5'-ACCAGTACATGTCGTTC			
55	GFM89FP	5'-AACCCGGTTCCGGATGTTC	50-60	120	Monomorphic
	GFM89RP	*5'-GGCGTCCTCTCGCTCGC*			
56	GFM90FP	5'-GGGCCTCGGTTTGCTGGG	50-60	150	Monomorphic
	GFM90RP	5'-GTTGCTGCAGTGCCTGCTG			
57	GFM94bFP	5'-GTCGTGTTGTAGCGGGC	50-60	152	Monomorphic
	GFM94bRP	5'-CTCTCACACTCTCATTCCTCG			
58	GFM99FP	5'-CCCCCTCGAAAAAACCCG	50-60	498	Monomorphic
	GFM99RP	5'-ACATGCTTCCTACACGGTTC			

Continued..

59	GFM101FP	5'-CTTTTTTTGGGGCCCGG	50	310	Monomorphic
	GFM101RP	5'-CTCTCTCGCACACAACAAC			
60	GFM102FP	5'-ATTCGGCACGAGGCTCTC	52.5	129	Monomorphic
	GFM102RP	5'-TTTAAGCCCCACATACCGAC			
61	GFM103FP	5'-GATTGTATAAGGCCCATCC	50	120	Monomorphic
	GFM103RP	5'-GGGACACACATCTCTCTC			
62	GFM104FP	5'-TTTTAAGGGGCCCCCCAG	52	600	Monomorphic
	GFM104RP	5'-TTTTTTGGTTTTCTCTGCGCG			
63	GFM105aFP	5'-GAGGATTTCTTTACCGTGGG	53	192	Monomorphic
	GFM105aRP	5'-CACCCGTAGGACATTCGG			
64	GFM105bFP	5'-GTGAGCGTGTAGAGAGTGG	53	284	Monomorphic
	GFM105bRP	5'-ATCTACTACACTCAGCACACAC			
65	GFM106FP	5'-AATTGGAACCAAATTCCCC	51	284	Monomorphic
	GFM106RP	5'- CACACACTCTCTCTCTCATCTC			
66	GFM107aFP	5'-CGGAGATCGTGGACAACGACC	57	206	Monomorphic
	GFM107aRP	5'-GCATGTCGAGGCCGCTGG			
67	GFM107bFP	5'-GCCCCCTTCTGAAGTGATGCTGAG	59	138	Monomorphic
	GFM107bRP	5'-GGTCGCAGCTGCAGTCCTG			
68	GFM108aFP	5'-GGAGGGCGGGGGGATAGAAA	59	135	Monomorphic
	GFM108aRP	5'-CCGCGCATGGTTGTTGTGACC			
69	GFM108bFP	5'-TGAGCGAGCGGTTGTGTG	54	133	Monomorphic
	GFM108bRP	5'-TCGCTCGCGTCTCTCGAC			
70	GFM109aFP	5'-GTAGATTAAAGAGCGCGTAGG	52.5	165	Monomorphic
	GFM109bRP	5'-CCCTCGCGGAAACATCTTC			
71	GFM109bFP	5'- AGAGAGAGGAGAGAGAGTA	48	165	Monomorphic
	GFM109bRP	5'-CTCTCTCTTCTTCTTCTC			
72	GFM110aFP	5'-AGGGAGTACATACGCAGGCG	57	173	Monomorphic
	GFM110aRP	5'-GGGAAATCCTCTGCACGCCC			
73	GFM110bFP	5'-AGTCAGACGTAGAGTGCGCG	56	175	Monomorphic
	GFM110bRP	5'-CAGTCTCACGGACATCTCGC			
74	GFM110cFP	5'-TAGAGAGAGGTGAGTCACGCAG	56	292	Monomorphic
	GFM110cRP	5'-ATCTGCACGCTGCCACTCG			
75	GFM111bFP	5'- AAGTAGAGAGAGAGTAGAGAGA	50	400	Monomorphic
	GFM111bRP	5'- CTCTCTTCTCATCTCTTCTAT			
76	GFM111cFP	5'- AAGTAGAGAGAGAGTAGAGAGA	52	400	Monomorphic
	GFM111cRP	5'- CTGCTTCTTCTTCTCACAACTC			
77	GFM111dFP	5'- AGAGAGTGAGAGAGGAGAGAGA	54	400	Monomorphic
	GFM111dRP	5'- CTCATCATATCTTCACTCCTCG			

Continued..

78	GFM112aFP	5'-AGGGCGAGGAAGCAAACGG	57	430	Monomorphic
	GFM112aRP	5'- CGCTCTCTCTACTCACTCTCCTCA			
79	GFM112bFP	5'-AGAGAGAGAGAGAGTGAGAAG	48	430	Monomorphic
	GFM112bRP	5'- TGCTCACACTCTACTCT			
80	GFM113FP	5'-GCACGAGGCCCCTTTCTCC	58	130	Monomorphic
	GFM113RP	5'-AGATCAGCGACTGCTGCCCC			
81	GFM114aFP	5'-GGAAGCAGAGACAGAAGCCC	56	356	Monomorphic
	GFM114aRP	5'-ACGCTAGCTCTACTACGTCTGA			
82	GFM114bFP	5'-GAGTAAGCGAGTCGTGAGC	52.5	500	Monomorphic
	GFM114bRP	5'-ACTCTCCTCTACTCTCTCTCT			
83	GFM115FP	5'-TAGTACGCAGAGCAAGAGAC	53	530	Monomorphic
	GFM115RP	5'-CTCTCTCTCTCTTCTCGCTTC			
84	GFM116FP	5'-TAAAGTATATAGGCAGGCGAGAGG	55	530	Monomorphic
	GFM116RP	5'-CTCTCTCTCTCTTACTCTCTCA			
85	GFM117aFP	5'-TGGGAGTAATCGGGACACTG	55	198	Monomorphic
	GFM117aRP	5'-CCAACGTAGAGCCATACCGG			
86	GFM117bFP	5'-TGTATGAGAGTGCGAGTGAG	52	400	Monomorphic
	GFM117bRP	5'-ACTCTCATGACTCTCAGCTC			
87	GFM118aFP	5'- AGAGAGAGAGGGAGGGGAGAG	57	120	Monomorphic
	GFM118aRP	5'-ATCCGGGGAACGATTGCCC			

Table 18 : Details of public domain Finger millet SSRs used in the study

S.N.	Primer name	Primer sequence	AT	PS (bp)	Remarks
1	FMUGEP1F	TTCAGTGGTGACGGAAGTTCT	58	233	Monomorphic
	FMUGEP1R	GGCTCCATGAAGAGCTTGAC			
2	FMUGEP3F	CCACGAGGCCATACTGAATAG	58	206	Monomorphic
	FMUGEP3R	GATGGCCACTAGGGATGTTG			
3	FMUGEP5F	TGTACACAACACCACACTGATG	58	215	Monomorphic
	FMUGEP5R	TTGTTTGGACGTTGGATGTG			
4	FMUGEP6F	AGCTGCAGTTTCAGTGGATTC	58	229	Polymorphic
	FMUGEP6R	TCAACAAGGTGAAGCAGAGC			
5	FMUGEP8F	ATTTCCGCCATCACTCCAC	58	297	Monomorphic
	FMUGEP8R	AGACGCAAATGGGTAAATGTC			
6	FMUGEP10F	AAACGCGATGAATTTTAAGCTC	58	400	Monomorphic
	FMUGEP10R	CTATGTCGTGTCCCATGTCG			
7	FMUGEP11F	CCTCGAGTGGGGATCCAG	58	153	Monomorphic
	FMUGEP11R	AAGACGCTGGTGGAAATAGC			
8	FMUGEP12F	ATCCCCACCTACGAGATGC	58	230	Monomorphic
	FMUGEP12R	TCAAAGTGATGCGTCAGGTC			
9	FMUGEP15F	AAGGCAATCTCGAATGCAAC	58	180	Polymorphic
	FMUGEP15R	AAGCCATGGATCCTTCCTTC			
10	FMUGEP18F	TTGCATGTGTTGCTTTTTGC	58	318	Monomorphic
	FMUGEP18R	TGTTCTTGATTGCAAACTGATG			
11	FMUGEP21F	CAATTGATGTCATTGGGACAAC	58	225	Monomorphic
	FMUGEP21R	GTATCCACCTGCATGCCAAC			
12	FMUGEP24F	GCCTTTTGATTGTTCAACTCG	58	183	Polymorphic
	FMUGEP24R	CGTGATCCCTCTCCTCTCTG			
13	FMUGEP26F	ATGGGGTTAGGGTTCGAGTC	58	227	Monomorphic
	FMUGEP26R	TGTCCCTCACTCGTCTCCTC			
14	FMUGEP31F	ATGTTGATAGCCGGAAATGG	58	241	Monomorphic
	FMUGEP31R	CCGTGAGCCTCGAGTTTTAG			
15	FMUGEP52F	TCATGCTAGCTTCAACACAACC	58	215	Monomorphic
	FMUGEP52R	TGCTGGGTGAAACCCTAGAC			
16	FMUGEP53F	TGCCACAACTGTCAACAAAAG	58	226	Polymorphic
	FMUGEP53R	CCTCGATGGCCATTATCAAG			
17	FMUGEP56F	CTCCGATACAGGCGTAAAGG	58	162	Polymorphic
	FMUGEP56R	ACCATAATAGGGCCGCTTG			
18	FMUGEP60F	AGCTCTGCTTGGTGGAGAAG	58	240	Monomorphic
	FMUGEP60R	TTTTCTACTGGTGGGCGAAG			

Continued..

19	FMUGEP65F	AGTGCTAGCTTCCCATCAGC	58	226	Monomorphic
	FMUGEP65R	ACCGAAACCCTTGTCAGTTC			
20	FMUGEP68F	CGGTCAGCATATAACGAATGG	58	232	Monomorphic
	FMUGEP68R	TCATTGATGAATCCGACGTG			
21	FMUGEP76F	GCACGTACGGATTCACATTG	58	168	Monomorphic
	FMUGEP76R	GGTACGGAGACATCGACACC			
22	FMUGEP77F	TTCGCGCGAAATATAGGC	58	245	Monomorphic
	FMUGEP77R	CTCGTAAGCACCCACCTTTC			
23	FMUGEP78F	AAGCAATCAACAAAGCCTTTTC	58	244	Monomorphic
	FMUGEP78R	TACAACGTCCAGGCAACAAG			
24	FMUGEP81F	AAGGGCCATACCAACACTCC	58	192	Monomorphic
	FMUGEP81R	CACTCGAGAACCGACCTTTG			
25	FMUGEP90F	GGCCTTTGCAGTCATGTGAG	58	232	Monomorphic
	FMUGEP90R	CGACTCCAGGTGTTGTTGG			
26	FMUGEP102F	ATGCAGCCTTTGTCATCTCC	58	184	Monomorphic
	FMUGEP102R	GATGCCTTCCTTCCCTTCTC			
27	FMUGEP104F	TCAGCACCACCTGAATAGG	58	189	Monomorphic
	FMUGEP104R	AATAGGGAGGGCGAAGACTC			
28	FMUGEP106F	AATTCCATTCTCTCGCATCG	58	175	Monomorphic
	FMUGEP106R	TGCTGTGCTCCTCTGTTGAC			
29	FMUGEP107F	TCATGCTCCATGAAGAGTGTG	58	224	Monomorphic
	FMUGEP107R	TGTCAAAAACCGGATCCAAG			
30	FMUGEP108F	GTTGGCTGCTCTGCTTATCC	58	150	Monomorphic
	FMUGEP108R	TATCTGCTTGTGCAGCTTCG			
31	FMUGEP110F	AAATTCGCATCCTTGCTGAC	58	192	Monomorphic
	FMUGEP110R	TGACAAGAGCACACCGACTC			

Table 19 : Details of public domain Rice SSRs used in the study

S.N.	Primers	AT	Prod Size	Remarks
1	RM 23F	55-60	200	Monomorphic
	RM 23R			
2	RM 26F	55-60	150	Monomorphic
	RM 26R			
3	RM 29F	55-60	250	Monomorphic
	RM 29R			
4	RM 39F	55-60	500	Monomorphic
	RM 39R			
5	RM 42F	55-60	300	Monomorphic
	RM 42R			
6	RM 47F	55-60	100	Monomorphic
	RM 47R			
7	RM 48F	55-60	200	Monomorphic
	RM 48R			
8	RM 51F	55-60	250	Monomorphic
	RM 51R			
9	RM 102F	55-60	311	Monomorphic
	RM 102R			
10	RM 105F	55-60	134	Monomorphic
	RM 105R			
11	RM 107F	55-60	300	Monomorphic
	RM 107R			
12	RM 111F	55-60	250	Monomorphic
	RM 111R			
13	RM 112F	55-60	150	Monomorphic
	RM 112R			
14	RM 115F	55-60	180	Monomorphic
	RM 115R			
15	RM 118F	55-60	350	Monomorphic
	RM 118R			
16	RM 119F	55-60	180	Monomorphic
	RM 119R			
17	RM 124F	55-60	300	Monomorphic
	RM 124R			
18	RM 132F	55-60	550	Monomorphic
	RM 132R			

Continued..

19	RM 135F	55-60	400	Monomorphic
	RM 135R			
20	RM 155F	55-60	200	Monomorphic
	RM 155R			
21	RM 159F	55-60	250	Monomorphic
	RM 159R			
22	RM 161F	55-60	187	Monomorphic
	RM 161R			
23	RM 162F	55-60	250	Monomorphic
	RM 162R			
24	RM 167F	55-60	390	Monomorphic
	RM 167R			
25	RM 179F	55-60	230	Monomorphic
	RM 179R			
26	RM 184F	55-60	230	Monomorphic
	RM 184R			
27	RM 185F	55-60	160	Monomorphic
	RM 185R			
28	RM 186F	55-60	124	Monomorphic
	RM 186R			
29	RM 190F	55-60	245	Monomorphic
	RM 190R			
30	RM 193F	55-60	290	Monomorphic
	RM 193R			
31	RM 195F	55-60	300	Monomorphic
	RM 195R			
32	RM 338F	55-60	210	Monomorphic
	RM 338R			
33	RM 339F	55-60	150	Monomorphic
	RM 339R			
34	RM 462F	55-60	150	Monomorphic
	RM 462R			
35	RM 312F	55-60	300	Monomorphic
	RM 312R			
36	RM 177F	45-55	219	Monomorphic
	RM 177R			
37	RM 1F	45-55	450	Monomorphic
	RM 1R			
38	RM 36F	45-55	300	Monomorphic
	RM 36R			

Continued..

39	RM 140F	45-55	400	Monomorphic
	RM 140R			
40	RM 145F	45-55	300	Monomorphic
	RM 145R			
41	RM 148F	45-55	250	Monomorphic
	RM 148R			
42	RM 154F	45-55	400	Monomorphic
	RM 154R			
43	RM 164F	45-55	250	Monomorphic
	RM 164R			
44	RM 168F	45-55	350	Monomorphic
	RM 168R			
45	RM 172F	45-55	200	Monomorphic
	RM 172R			
46	RM 174F	45-55	300	Monomorphic
	RM 174R			
47	RM 207F	45-55	200	Monomorphic
	RM 207R			
48	RM 214F	45-55	300	Monomorphic
	RM 214R			
49	RM 216F	45-55	150	Monomorphic
	RM 216R			
50	RM 220F	45-55	220	Monomorphic
	RM 220R			
51	RM 225F	45-55	200	Monomorphic
	RM 225R			
52	RM 229F	45-55	200	Monomorphic
	RM 229R			
53	RM 230F	45-55	200	Monomorphic
	RM 230R			
54	RM 232F	45-55	400	Monomorphic
	RM 232R			
55	RM 236F	45-55	400	Monomorphic
	RM 236R			
56	RM 244F	45-55	200	Monomorphic
	RM 244R			
57	RM 247F	45-55	200	Monomorphic
	RM 247R			
58	RM 248F	45-55	200	Monomorphic
	RM 248R			

Continued..

59	RM 249F	45-55	150	Monomorphic
	RM 249R			
60	RM 250F	45-55	350	Monomorphic
	RM 250R			
61	RM 251F	45-55	200	Monomorphic
	RM 251R			
62	RM 254F	45-55	130	Monomorphic
	RM 254R			
63	RM 258F	45-55	280	Monomorphic
	RM 258R			
64	RM 259F	45-55	310	Monomorphic
	RM 259R			
65	RM 261F	45-55	300	Monomorphic
	RM 261R			
66	RM 263F	45-55	200	Monomorphic
	RM 263R			
67	RM 279F	45-55	300	Monomorphic
	RM 279R			
68	RM 280F	45-55	400	Monomorphic
	RM 280R			
69	RM 283F	45-55	180	Monomorphic
	RM 283R			

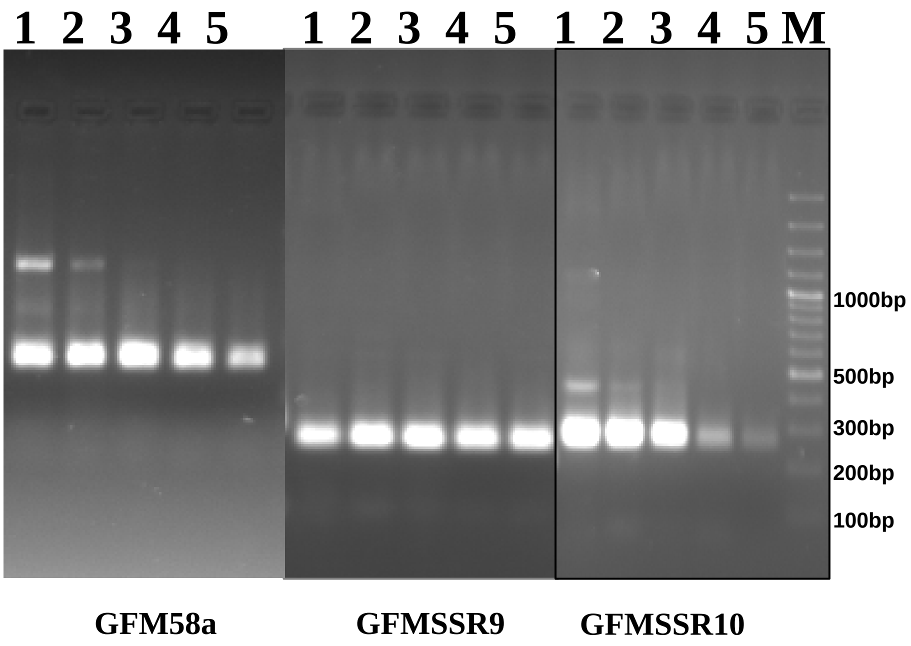


Plate 5 : Standardization of annealing temperature of genic SSR primers for PCR amplification

The genic SSR primers were initially standardized for annealing temperature at different gradient temperatures. The gel showing few SSR primers amplification and their standardized annealing temperature are given Lane 1-55.0^0C, 2-56.7^0C, 3-59.1^0C, 4-61.7^0C, 5-64.6^0C; M-Marker (100bp)

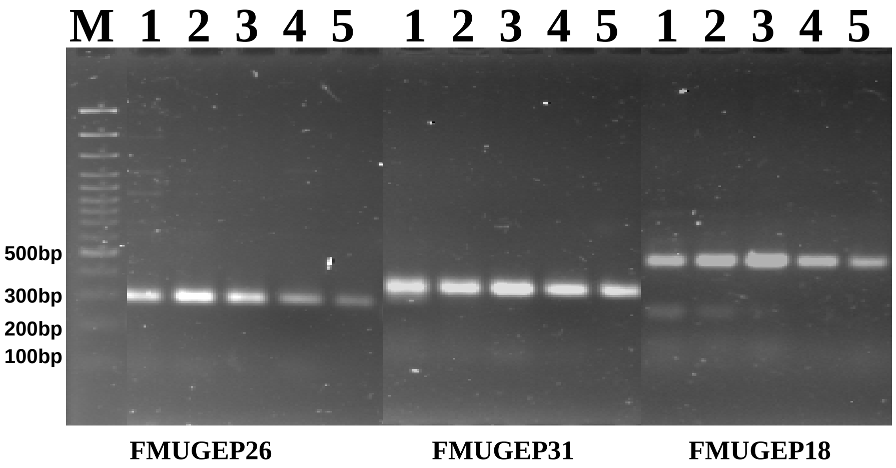


Plate 6 : Public domain fingermillet SSRs showing locus specificity

Finger millet SSRs from public domain* also checked for their locus specific amplification in mapping population The gel depicts standardization of annealing temperature of few SSR primers Lane 1-55.0^0C, 2-56.7^0C, 3-59.1^0C, 4-61.7^0C, 5-64.6^0C; M-Marker (100bp)

***Mathews M. Dida · Srinivasachary · Sujatha Ramakrishnan ·JeVrey L. Bennetzen · Mike D. Gale · Katrien M. Devos** Theor Appl Genet (2007) 114:321–332

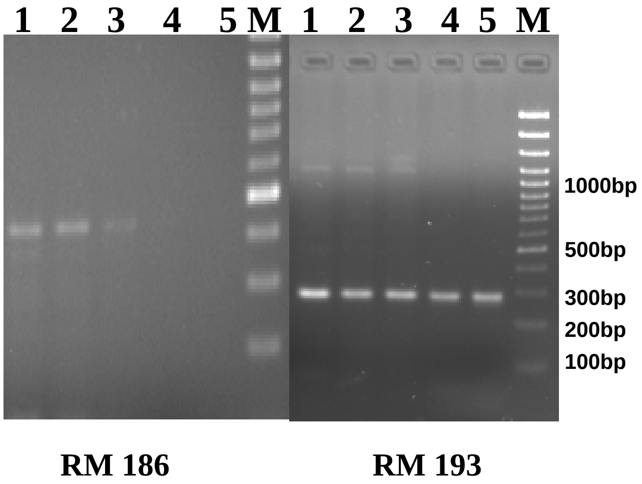


Plate 7 : Public domain rice SSRs showing locus specificity

Rice SSRs from public domain also checked for their locus specific amplification in mapping population The gel depicts standardization of annealing temperature of few SSR primers Lane 1-55.0^{0}C, 2-56.7^{0}C, 3-59.1^{0}C, 4-61.7^{0}C, 5-64.6^{0}C; M-Marker (100bp)

Several drought tolerance traits such as root traits, $\Delta^{13}C$ including growth parameters were recorded to examine the differences between the parents as well as among the RILs. The parent IE 2912 emerged as the superior parent with higher values recorded for all parameters except SCMR (Table 20). Significant difference found among parental lines in leaf traits such as total leaf area (3686 cm^2 pl^{-1} in IE 2885 and 5884 cm^2 pl^{-1} in IE 2912 with P=0.005) and leaf weight (15.5 g pl^{-1} in IE 2885 and 23.2 g pl^{-1}in IE 2912 with P=0.002).

Though, tiller number per plant was not significantly different between the parents, stem weight (56.6 g pl^{-1} in IE 2912 and 45.6 g pl^{-1} in IE 2885) and plant height (141.5 cm in IE 2912 and 127.7 cm in IE 2885) varied significantly. The two parents differed significantly in root traits such as root dry weight (P=0.04) and root volume (P=0.006) with parent IE 2912 having the higher root dry weight (16.6 g pl^{-1}), root volume (17.6 cm^3) compared with IE 2885. Root length, root to shoot and root to leaf area ratios were not significant among parents.

Total dry matter varied significantly (P=0.001) between parents with IE 2912 having higher total dry matter (96.3 g pl^{-1}) than IE 2885 (70.0 g pl^{-1}). Besides root traits, one other trait that has significant influence on drought tolerance is water use efficiency (WUE). The parameter was estimated using carbon isotope discrimination ($\Delta^{13}C$) as a surrogate. Results revealed that the parental lines of the mapping population did not differ significantly. Water use efficiency is the ratio of total biomass to the water used. Factors that independently influence either biomass accumulation or total transpiration can potentially alter WUE. Therefore, though WUE does not show differences, it is quite likely that two parental lines differ in factors that regulate WUE.

4.5 Phenotypic evaluation of mapping population

The mapping population (recombinant inbred lines) was also subjected to physiological characterization in addition to parental lines such as leaf, shoot and root traits along with SPAD chlorophyll meter reading (SCMR), moisture retention capacity (MRC), carbon isotope discrimination ($\Delta^{13}C$) and total dry matter.

Table 20 : Differences in physiological traits among parental lines in finger millet during first season (n=3)

Trait	IE 2885	IE 2912	P < 0.05
Total leaf area (cm^2 pl^{-1})	3686	5884	0.005
Leaf weight (g pl^{-1})	15.5	23.2	0.002
Specific leaf area (cm^2g-^{1})	237.5	254	NS
SCMR	47.9	42.9	NS
Stem weight (g pl^{-1})	45.6	56.6	0.002
Plant height (cm)	127.7	141.5	0.005
Tiller number per plant	3.8	4.3	NS
Root length (cm)	30.6	42.1	NS
Root volume (cm^3)	14.4	17.6	0.006
Root dry weight (g pl^{-1})	8.9	16.6	0.044
Root/Shoot	0.145	0.208	NS
Root/LA (g cm^{-2})	0.0024	0.0028	NS
Total dry matter (g pl^{-1})	70.0	96.3	0.001
Moisture retention capacity (%)	98.6	97.54	NS
$\Delta^{13}C$ (‰)	5.71	5.36	NS

4.5a Phenotypic variations for leaf traits in the RILs of finger millet

All the leaf traits like, total leaf area, total leaf weight and SLA showed significant variability among the RILs. The total leaf area varied from 2143 $cm^2 plant^{-1}$ in MLC 51-3 to 8879 $cm^2.plant^{-1}$ in MLC 62-3 with a mean of 4758 $cm^2.plant^{-1}$ (Table 21). The total leaf weight also showed a significant variability ranging from 7.75 $g.plant^{-1}$ in MLC 74-3 to 37.06 $g.plant^{-1}$ in MLC 148-1. The ratio of leaf area to leaf weight referred to as Specific Leaf Area (SLA) varied between 135.6 cm^2 g^{-1} in MLC 85-4and 301.5 cm^2 g^{-1} in MLC 83-1 representing a significant genotypic variability. All the leaf traits showed normal distribution around the mean and showed continuous variation (Fig.8) indicating that the traits are quantitative in nature. Total leaf area, leaf weight and SLA were positively skewed indicating the predominance of transgressive segregants towards the superior parent for these traits in the population**.** The sharper kurtosis peaks for LA and SLA (0.55 and 1.37, respectively) indicates that the more segregants had a value much higher than the mean of the population, whereas leaf weight showed negative kurtosis (-0.004).

4.5b Phenotypic variations for shoot traits in the RILs of finger millet

Several parameters associated with shoot growth such as plant height, number of tillers and shoot weight were recorded. The tiller number varied significantly from 1.2 in MLC 71-3 to 6.0 in MLC 68-2 (Table 21). RIL MLC 2-2 showed the lowest stem weight of 31.77 $g.plant^{-1}$ and RIL MLC 43-2 showed a highest stem weight of 131.68 $g.plant^{-1}$. The mean plant height of RILs was 144 cm ranging from 118.3 cm in MLC 17-3 to 161.6 cm in MLC 41-3 representing a significant variability.

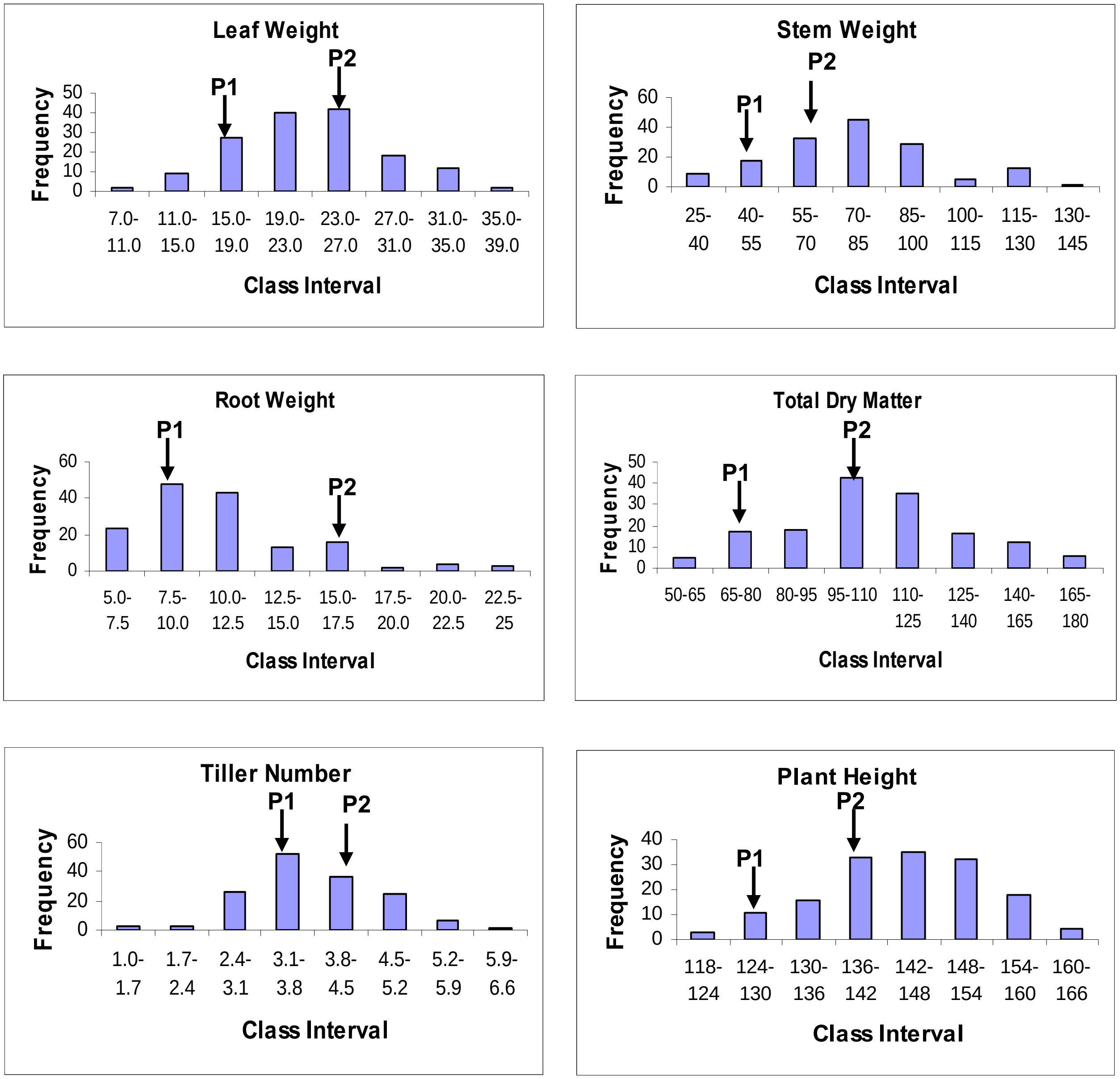
The shoot traits exhibited normal distribution as indicated in Figure 8. Stem weight (0.41) and tiller number (0.08) were positively skewed whereas plant height was negatively skewed (-0.40). Both stem weight (-0.07) and plant height (-0.23) revealed negative kurtosis whereas tiller number showed positive kurtosis (0.20)

Table 21 : Genetic variability of physiological traits among recombinant inbred lines of the mapping population (IE 2912xIE 2885) in finger millet in first season

Trait	**Minimum**	**Maximum**	**Mean**	**P value**	**SEM**	**$CD_{0.05}$**	**CV %**	**Kurtosis**	**Skewness**
Total leaf area (cm^2 pl^{-1})	2143	8879	4758	**	10.34	10.80	10.09	0.55	0.60
Leaf weight (g pl^{-1})	7.75	37.06	23.07	**	0.44	0.51	9.77	-0.004	0.12
Specific leaf area ($cm^2 g$-^{1})	135.60	301.50	207.10	**	2.12	3.51	7.47	1.37	0.29
SCMR	40.13	50.48	46.50	NS	0.16	0.68	6.46	0.63	-0.52
Stem weight (g pl^{-1})	31.77	131.68	75.18	**	1.86	1.39	8.17	-0.07	0.41
Plant height (cm)	118.3	161.6	144	**	0.73	2.45	7.54	-0.23	-0.40
Tiller number per plant	1.2	6.0	3.85	**	0.07	0.18	21.34	0.20	0.08
Root length (cm)	25.88	63.91	44.80	**	0.67	1.12	11.03	0.20	-0.26
Root volume (cm^3)	6.5	52.5	21.2	**	0.78	0.44	9.18	2.29	1.46
Root dry weight (g pl^{-1})	5.26	24.16	11.15	**	0.30	0.46	18.37	1.42	1.17
Root/Shoot	0.03	0.18	0.10	**	0.002	0.004	16.5	0.38	0.64
Root/LA (g cm^{-2})	0.009	0.075	0.024	**	0.007	0.001	21.37	9.54	2.38
Total dry matter (g pl^{-1})	53.4	174.7	109.42	**	2.18	1.67	6.75	-0.071	0.34
Moisture retention capacity (%)	85.3	99.1	96.77	NS	0.12	0.41	1.91	22.52	-3.57
$\Delta^{13}C$ (‰)	4.79	5.99	5.41	NS	0.02	0.08	6.87	0.2	-0.14

****significant at 5 %**
NS-Non significant

Along with parents mapping population was also phenotyped most of the traits revealed significant genetic variability except $\Delta^{13}C$, SCMR and MRC revealed no significant variability in the mapping population.



P1-IE 2885
P2-IE 2912

Continued ..

Fig. 8 : Frequency distribution of physiological traits in the first season experiment

4.5c Phenotypic variations for root traits in the RILs of finger millet

Water acquisition from deeper soil profiles is a function of canopy leaf area and the ability of root traits to harness water from the soil. In the present study, genotypic variability in several parameters associated with the roots was ascertained. The RILs had a mean root length of 44.8 cm and mean root weight was 11.15 g plant^{-1}. The lowest root weight of 5.26 g plant^{-1} was noticed in RIL MLC 41-3 and the highest was noticed in MLC 54-4 (24.76 g plant^{-1}). The root to shoot ratio also varied significantly from 0.03 in MLC 5-5 to 0.18 in MLC 74-3 (Table 21). The frequency distribution of root traits showed continuous variability confirming the polygenic inheritance of the trait (Fig.8). Except root length (-0.26) other root traits such as root volume (1.46), root weight (1.17) root to shoot ratio (0.80) and root to leaf area (2.38) were positively skewed with high kurtosis indicating that majority of the recombinant inbred lines performed better than the superior parents for these traits. The kurtosis for root to leaf area (9.54) was maximum among the root traits.

The total biomass (TBM) or total dry matter in the population varied significantly around the mean of 109.42 g pl^{-1} ranging between 53.40 g pl^{-1} (MLC 2-2) and 174.7 g pl^{-1} in MLC 98-1which is normally distributed with positive skewness (0.34) and negative kurtosis (-0.07).

Other traits such as Δ^{13}C, SCMR and MRC revealed no significant variability in the mapping population.

4.6 Physiological traits that explain the observed variations in Total Biomass (TBM)

The total biomass is the sum of the root and shoot dry matter produced by a plant. Further, the biomass produced by a plant is known to be influenced by genetic makeup of the plant, its physiological behavior as well as the external influences like environmental factors. Several generic models have been proposed to explain the observed variations in BM. Among these Duncan's model and Pasioura's model are widely accepted. As per the Duncan's model BM is the function of LA and NAR, while Passioura suggested that BM is dependent on the variations in WUE and Transpiration (Root traits). Hence, examining the variations in these traits and assessing the influence on BM is crucial in crop improvement efforts. To decipher the influence of these important physiological traits, the recombinant inbred lines were classified based on different approaches. The results are presented in the following section

4.7 Classification of Recombinant inbred lines based on total biomass (BM)

Of the 150 RILs examined in the investigation, 20 genotypes each representing extreme values of BM were identified. The biomass of the high total dry matter category was 3 fold higher than the low total dry matter category. The mean BM of low BM types was 67.7 g.plant^{-1}, while it was 158.1 g.plant^{-1} for high BM types (Table 22). Similarly leaf weight ,leaf area, root weight, root volume and shoot weight were significantly higher in the high BM category To ascertain the influence of several parameters on BM, correlation coefficient for the relationship between BM and these traits were determined separately for the genotypes coming under high and low BM types (Table 23). In both high and low BM genotypes shoot weight root weight and leaf area, showed significant positive relationship with BM, whereas $\Delta^{13}C$, a measure of WUE was not related with BM.

4.8 Classification of Recombinant inbred lines based on total biomass (BM) with similar leaf area

Assessment of contribution of physiological traits other than the canopy cover that determine the variability in BM is best done when genotypes with similar leaf area but differing in BM are compared. The genotypes that had a mean leaf area of around 4800 cm^{2} pl^{-1} were selected in both low and high BM groups. The two groups despite little differences in leaf area, differed significantly for BM (Table 24). The high biomass groups had 134.65 g.plant^{-1} of BM, while it was 82.88 g.plant^{-1} in low biomass group, representing a 62 % difference. Traits such as root weight, root volume, shoot weight and leaf weight were found significantly higher in high BM types than low BM types which contributed to differences in BM. There was no significant difference in $\Delta^{13}C$ between low and high BM types.

Table 22 : Differences in various physiological traits among the Low and High Biomass groups of RILs

Trait	Low BM(g pl^{-1})	High BM (g pl^{-1})	% Increase in High BM types
Root Weight (g pl^{-1})	7.8	14.0	79.4
Shoot Weight (g pl^{-1})	59.8	144.0	140.8
Total leaf area (cm^2 pl^{-1})	3553	5521	55.3
Total Biomass (g pl^{-1})	67.7	158.1	133.5
Specific leaf area (cm^{2}g-^{1})	201.8	202.9	0.7
Δ^{13}C (‰)	5.39	5.45	1.1
Root volume (cm^3)	16.1	26.0	61.4
Leaf weight (g pl^{-1})	17.6	27.2	54.5

From the mapping population 20 RILs representing extreme values were identified and classified as low and high biomass types among them different physiological traits have been analysed

Table 23 : Correlation values for various physiological traits among the Low and High Biomass groups of RILs

Traits	Low BM types (r values)	High BM types (r values)
Δ^{13}C Vs BM	(-0.123) NS	(-0.098)NS
Shoot wt Vs BM	0.952	0.923
Root wt Vs BM	0.342	0.171
Leaf area Vs BM	0.281	0.434

From the mapping population 20 RILs representing extreme values were identified and classified as low and high biomass types among them different physiological traits have been correlated with biomass

Table 24 : Differences in various physiological traits between the two groups of recombinant inbred lines of fingermillet with similar Leaf Area but differing in Biomass

Trait	Low BM ($g\ pl^{-1}$)	High BM ($g\ pl^{-1}$)	% Increase in High BM types
Root Weight ($g\ pl^{-1}$)	9.48	12.99	37.02
Stem Weight ($g\ pl^{-1}$)	52.60	96.12	82.37
Total leaf area ($cm^2\ pl^{-1}$)	4803	4935	2.70
Total Biomass ($g\ pl^{-1}$)	82.88	134.65	62.46
$\Delta^{13}C$ (‰)	5.37	5.48	2.00
Root volume (cm^3)	17.10	27.30	59.60
Leaf weight ($g\ pl^{-1}$)	20.80	25.54	22.78

From the mapping population 20 RILs representing extreme values were identified and classified as low and high biomass types which are having similar leaf area, among them different physiological traits have been analysed

Table 25 : Differences in various physiological traits between the two groups of recombinant inbred lines of fingermillet with similar Leaf Area but differing in $\Delta^{13}C$

Trait	Low $\Delta^{13}C$ (‰)	High $\Delta^{13}C$ (‰)	% Increase in High $\Delta^{13}C$ types
Root Weight ($g\ pl^{-1}$)	11.0	12.0	9.0
Shoot Weight ($g\ pl^{-1}$)	93.1	99.2	6.5
Total leaf area ($cm^2\ pl^{-1}$)	4506	4550	0.9
Total Biomass ($g\ pl^{-1}$)	104.1	111.2	6.8
Specific leaf area	210.5	119.6	-5.0
$\Delta^{13}C$ (‰)	4.96	5.79	16.7
Root volume (cm^3)	21.7	24.2	11.5
Leaf weight ($g\ pl^{-1}$)	21.4	22.8	5.1

From the mapping population 20 RILs representing extreme values were identified and classified as low and high **$\Delta^{13}C$** types which are having similar leaf area, among them different physiological traits have been analysed

4.9 Classification of Recombinant inbred lines based on $\Delta^{13}C$ with similar leaf area

Recombinant inbred lines with similar leaf leaf area were also classified as low and high $\Delta^{13}C$ types and compared (Table 25), but all the traits did not show significant variation between low and high $\Delta^{13}C$ types. In low $\Delta^{13}C$ types, shoot weight (r=0.971) and root weight(r=0.319) are positively, significantly correlated with biomass but there was no relationship between leaf area and biomass. Whereas, $\Delta^{13}C$ and biomass was negatively correlated (r=-0.281). In high $\Delta^{13}C$ types, shoot weight(r=-0.505), leaf area(r=-0.742) and root weight (r=-0.613) are negatively correlated with biomass whereas, correlation of $\Delta^{13}C$ and biomass was positive and significant (r=0.325) which are in contrast with low $\Delta^{13}C$ types (Table 26).

4.10 Relationship between biometric traits in the mapping population of finger millet

Being quantitative in nature, the physiological and biometric traits recorded in the mapping population are influenced not only by the environmental factors but also the plant's genetic constitution. Hence, it is important to know the interrelationship among these traits that contribute to the total biomass of the plant when breeding programme targets yield improvement. As expected, a positive correlation was observed between various root, shoot associated traits and the total biomass but root to shoot ratio showed a negative relationship with biomass (Table 27). Apart from root and shoot dry weight, a strong positive correlation was observed between the leaf area and the biomass (Fig 9) suggesting that the photosynthetic surface area contributes significantly to the biomass production. A significant positive correlation between root and shoot dry weight (Fig 10) indicated that the root biomass significantly contributes to the shoot growth. However an inverse relationship was observed between root to shoot ratio and the biomass suggesting that plant allocate carbon reserve to root at the cost of shoot growth (Fig 11). Though a strong positive association was noticed between leaf area and biomass, $\Delta^{13}C$ did not show any relationship with biomass (Fig 12) indicating that the increase in biomass could be achieved either through increased transpiration associated with leaf area and root traits or

Table 26 : Correlation coefficients for various physiological and biometric traits among the Low and High $\Delta^{13}C$ groups of recombinant inbred lines of fingermillet

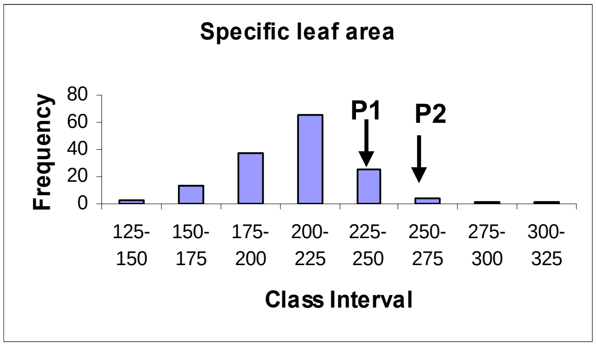
Trait	Low $\Delta^{13}C$ (‰) Types (r values)	High $\Delta^{13}C$ (‰) Types (r values)
Root Weight Vs BM	0.319	-0.505
Shoot Weight Vs BM	0.971	-0.742
Leaf Area Vs BM	0.078	-0.613
$\Delta^{13}C$ Vs BM	-0.281	0.325

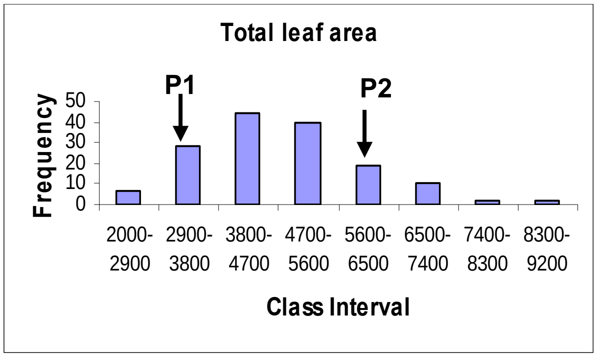
From the mapping population 20 RILs representing extreme values were identified and classified as low and high $\Delta^{13}C$ types among them different physiological traits have been correlated with biomas

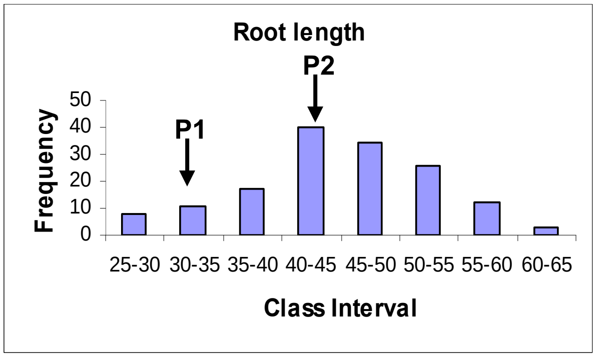
Table 27 : Correlation coefficients of different root and shoot traits with total biomass in the recombinant inbred lines of finger millet

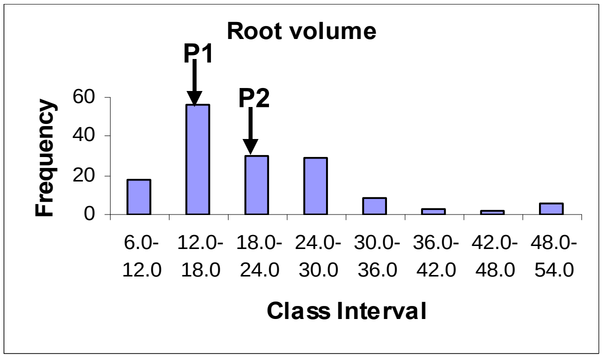
Traits	r Values
Root volume Vs BM	0.274
Root length Vs BM	0.177
Root dry weight Vs BM	0.542
R/S Vs BM	-0.228
Tiller number Vs BM	0.314
Shoot dry weight Vs BM	0.960
Leaf Area Vs BM	0.443
Leaf weight Vs BM	0.512

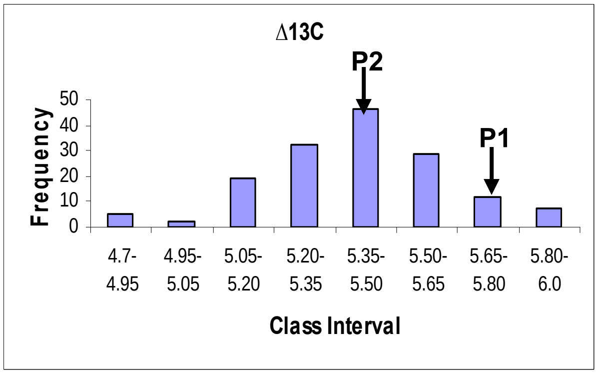
Physiological traits recorded in the mapping population are influenced not only by the environmental factors but also the plant's genetic constitution. Hence, the interrelationship among these traits that contribute to the total biomass of the plant has been ascertained through correlation analysis

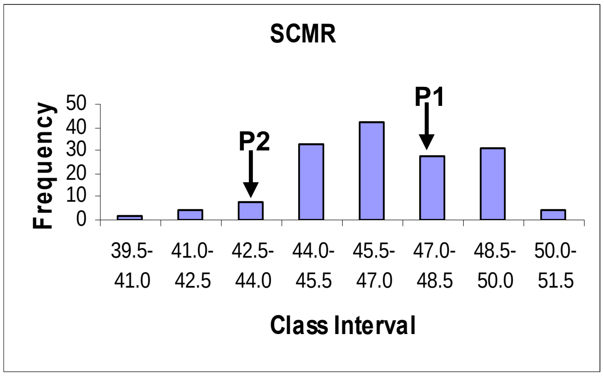


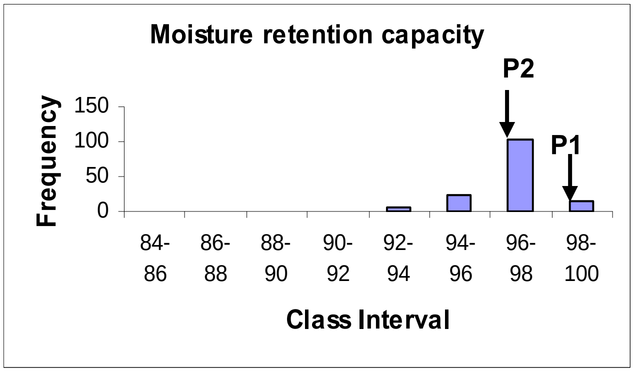












P1-IE 2885
P2-IE 2912

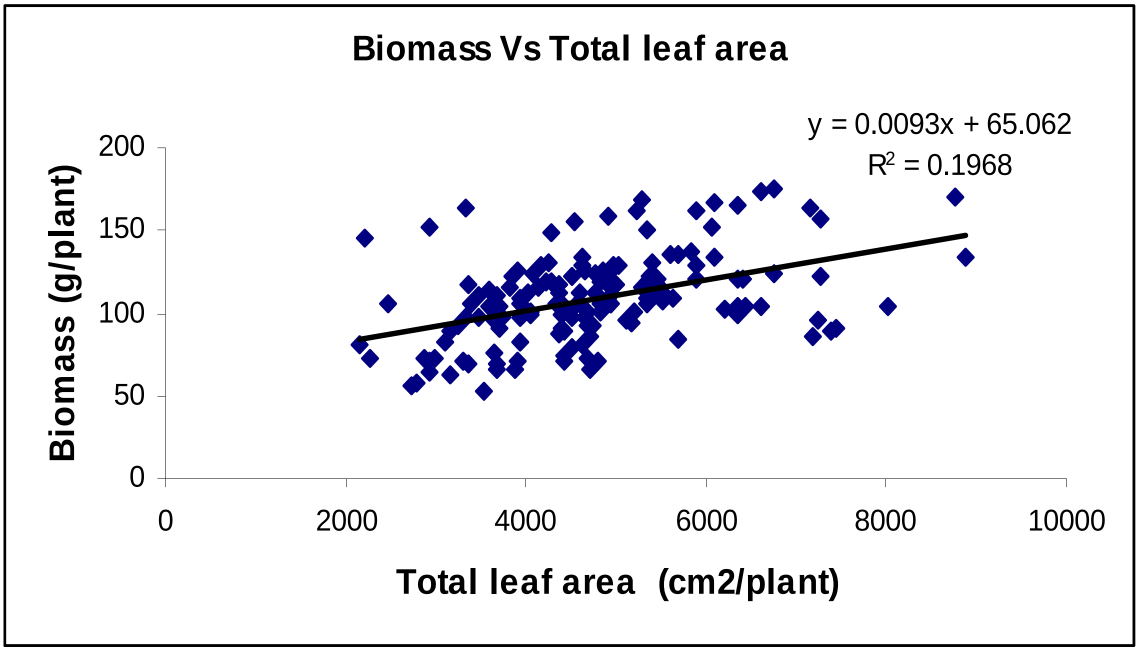


Fig. 9 : Relationship between Leaf area (dm^2 plant^{-1}) and Biomass (g plant^{-1}) among RILs of finger millet

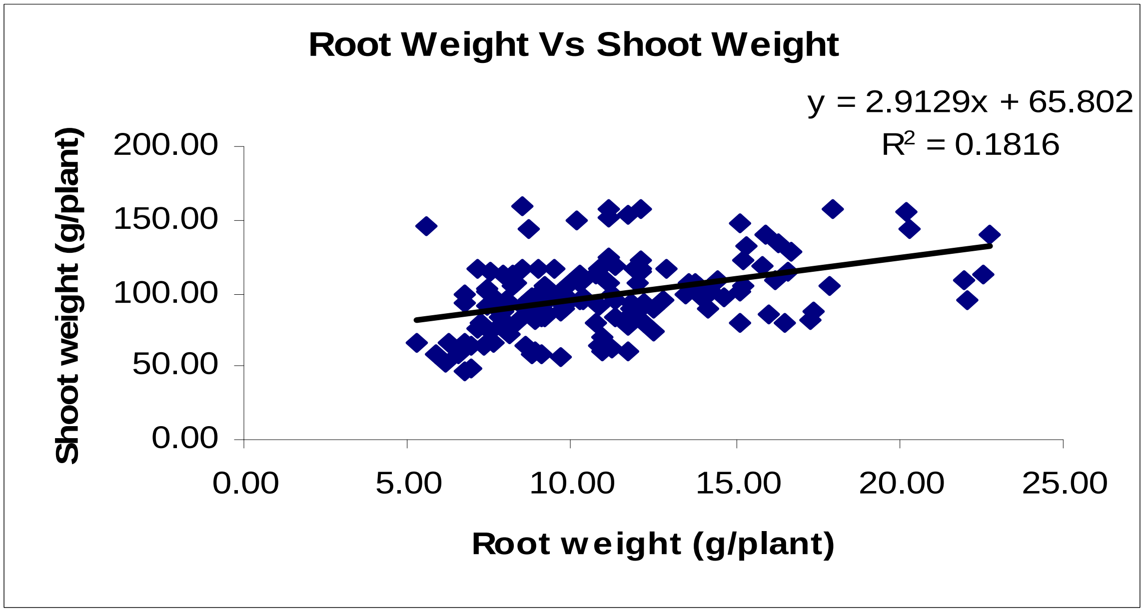


Fig. 10 : Relationship between Root weight (g plant^{-1}) and Shoot weight (g plant^{-1}) among RILs of finger millet

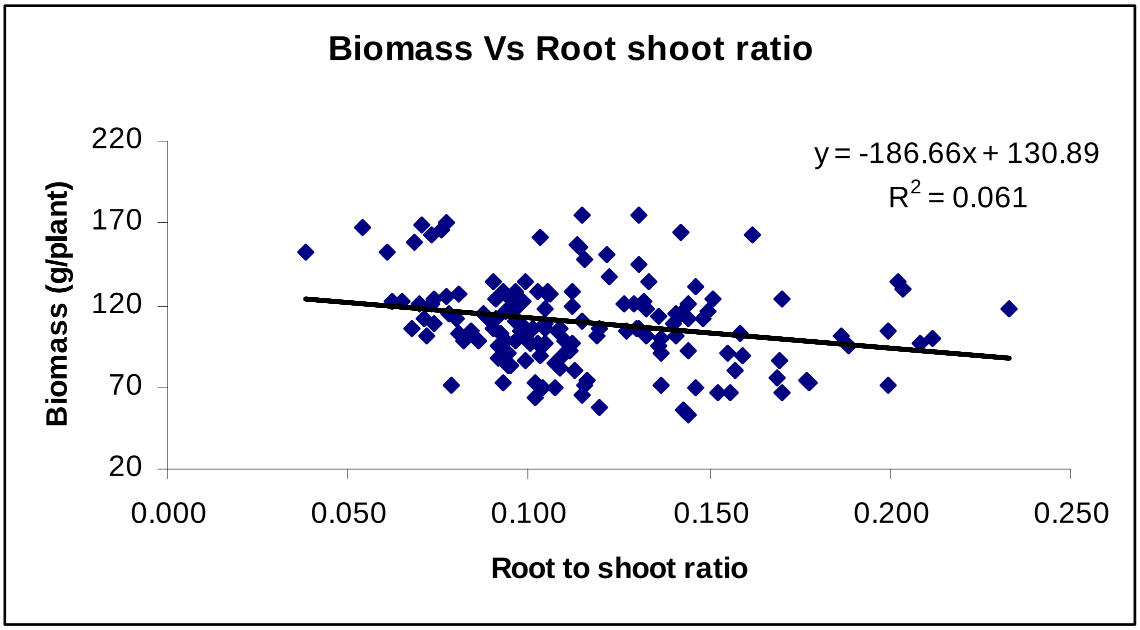


Fig. 11: Relationship between Biomass (g plant^{-1}) and Root to shoot ratio among RILs of finger millet

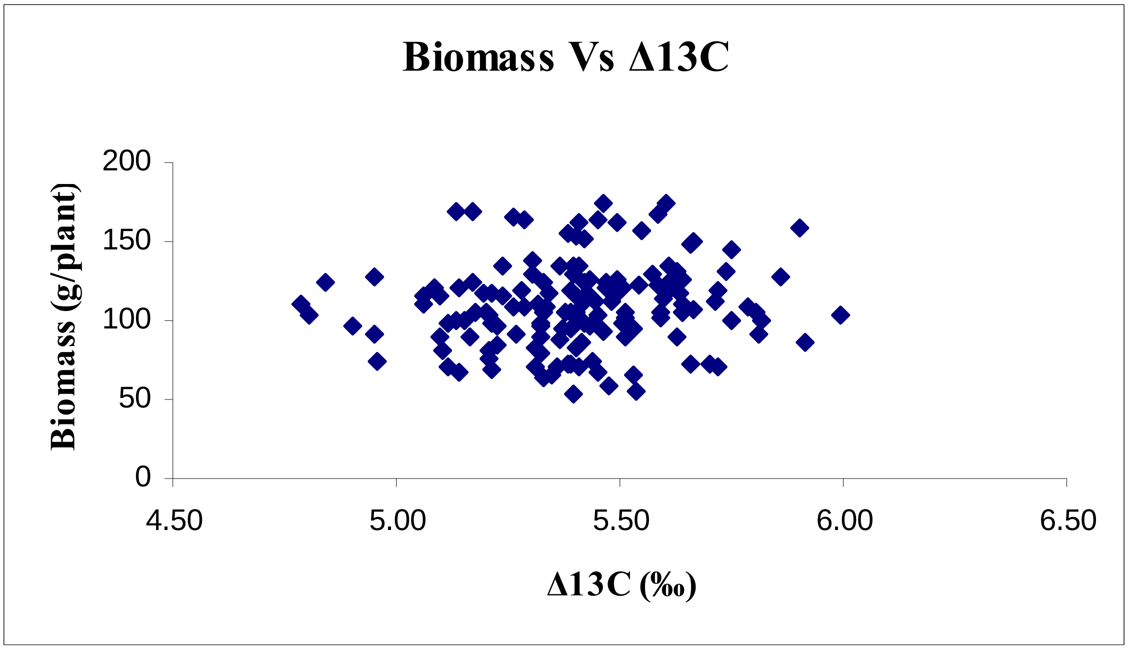


Fig. 12 : Relationship between Biomass (g plant^{-1}) and Δ^{13}C (‰) among RILs of finger millet

though superior water use efficiency. Thus, it is possible that high $\Delta^{13}C$ types are also associated with high root biomass..

4.11 Genetics of traits among the RILs of finger millet

The phenotypic characterization of the mapping population revealed that significant variability exists in several parameters distributed normally around the mean. Among these traits LA was the most prominent contributor to BM. However, at a given leaf area, physiological traits like WUE and other biometric parameters like root weight significantly contributed to the BM. However, it would become essential to assess the heritability of these traits before attempting to exploit these traits in breeding program. An effort was made to assess the genetics of these traits.

4.11a Estimation of GCV, PCV, GA and heritability for biometric traits

The effectiveness of phenotypic selection would largely depend on the genetic control of the trait. Therefore, to know the extent of genotype and environmental effect on phenotype the genetic parameters like Genotypic coefficient of variation (GCV), Phenotypic coefficient of variation (PCV), Broad sense heritability (H^2) and the Genetic advance (GA) as per cent mean were calculated for biometric traits

4.11b Genetic parameters for biometric traits

Heritability denotes the phenotypic variance caused due to genotype. All of the biometric traits showed a high heritability except plant height (17.48%) tiller number and which showed a moderate heritability of 32.60 per cent (Table 28). Highest heritability was noticed in root volume (95.97%) followed by $\Delta^{13}C$ (94.8%). The GCV and PCV for all the traits were moderate to high except for SCMR, $\Delta^{13}C$ and tiller number for which both GCV(4.18, 7.90 and 3.47 respectively) and PCV were low (4.98, 8.17 and 8.3, respectively). Highest GCV was noticed for root volume (44.8) and lowest GCV for plant height (3.47). PCV for the biometric traits ranged from 4.31 % for MRC to 45.7 % root volume. Genetic advance (GA) as per cent mean was high for all the biometric traits except for SCMR, MRC and plant height. Moderate Genetic advance (GA) as per cent mean was noticed in SLA, $\Delta^{13}C$ and tiller number per plant.

Table 28 : Genetic parameters for biometric traits among the RILs of the cross IE2912 x IE 2885 of finger millet

Trait	Heritability (%)	GCV (%)	PCV (%)	Genetic advance as per cent mean
SCMR	70.60	4.18	4.98	7.24
Total Leaf Area ($dm^2 pl^{-1}$)	86.75	25.81	27.71	49.52
Specific leaf area($g\ cm^{-2}$)	69.92	11.39	13.63	19.63
$\Delta^{13}C$ (‰)	94.80	7.90	8.17	15.91
Moisture Retention Capacity (%)	69.00	3.58	4.31	6.12
Leaf weight ($g\ pl^{-1}$)	84.52	22.84	24.84	43.21
Stem weight ($g\ pl^{-1}$)	92.99	29.76	30.86	59.85
Root weight ($g\ pl^{-1}$)	74.38	31.33	36.33	55.61
Root volume (cm^3)	95.97	44.80	45.73	90.37
Root length (cm)	69.38	16.60	19.93	23.10
Plant height (cm)	17.48	3.47	8.30	2.98
Tiller number	32.60	14.84	26.00	17.46
Biomass ($g\ pl^{-1}$)	92.66	24.00	24.93	47.59

II Experiment

4.12 Phenotypic evaluation of parents

It is well known that many physiological traits/ drought traits are quantitative in nature revealing continuous variation. For successful identification of relevant QTLs/markers which are associated with physiological traits, assessing the consistency of the traits is important. In the second experiment, representative number of recombinant inbred lines (100) was used for assessing the stability of the traits.

Most of the traits were better in the parent IE 2912 than IE 2885. (Table 29). Significant difference found among parental lines in leaf traits such as leaf weight (18.56 g pl^{-1} in IE 2885 and 27.1 g pl^{-1}in IE 2912 with P=0.005). Among shoot traits, tiller number showed no significant difference between the parents, whereas higher stem weight was observed in IE 2912 (69.07 g pl^{-1}), compared to IE 2885 (54.12 g pl^{-1}) and plant height also varied significantly (140.42 cm in IE 2912 and 102.67 cm in IE 2885 with P=0.001).

The parents showed significant difference in root traits such as root dry weight (P=0.001) and root volume (P=0.002), wherein parent IE 2912 revealed higher root dry weight (22.69 g pl^{-1}), root volume (24.72 cm^3) and low root dry weight (9.23 g pl^{-1}), root volume (14.89 cm^3) was noticed in IE 2885. Root length (50 cm in IE 2912 and 36.89 cm in IE 2885 with P=0.005), root to shoot ratio (0.24 in IE 2912 and 0.13 in IE 2885 with P=0.005) were also found to be significant among parents.

Total dry matter varied significantly (P=0.002) between parents with IE 2912 recording higher total dry matter of 118.83 g pl^{-1} compared to IE 2885 (81.9 g pl^{-1}).

4.13 Phenotypic evaluation of mapping population

4.13a Phenotypic variations for leaf, shoot and root traits in the RILs of finger millet

The total leaf weight also showed a significant variability ranging from 8.94 $g.plant^{-1}$ in MLC 81-3 to 38.42 $g.plant^{-1}$ in MLC 7-4 (Table 30) which was normally distributed indicating that the traits are quantitative in nature (Fig 13). Leaf weight was

Table 29 : Differences in physiological traits among parental lines in finger millet during second season (n=3)

Trait	IE 2885	IE 2912	$P < 0.05$
Leaf weight (g pl^{-1})	18.56	27.10	0.005
Stem weight (g pl^{-1})	54.12	69.07	0.005
Plant height (cm)	102.67	140.42	0.001
Tiller number	3.54	4.17	NS
Root length (cm)	36.89	50.00	0.005
Root volume (cm^3)	14.89	24.72	0.002
Root dry weight (g pl^{-1})	9.23	22.69	0.001
Root/Shoot	0.13	0.24	0.005
Total dry matter (g pl^{-1})	81.9	118.83	0.002

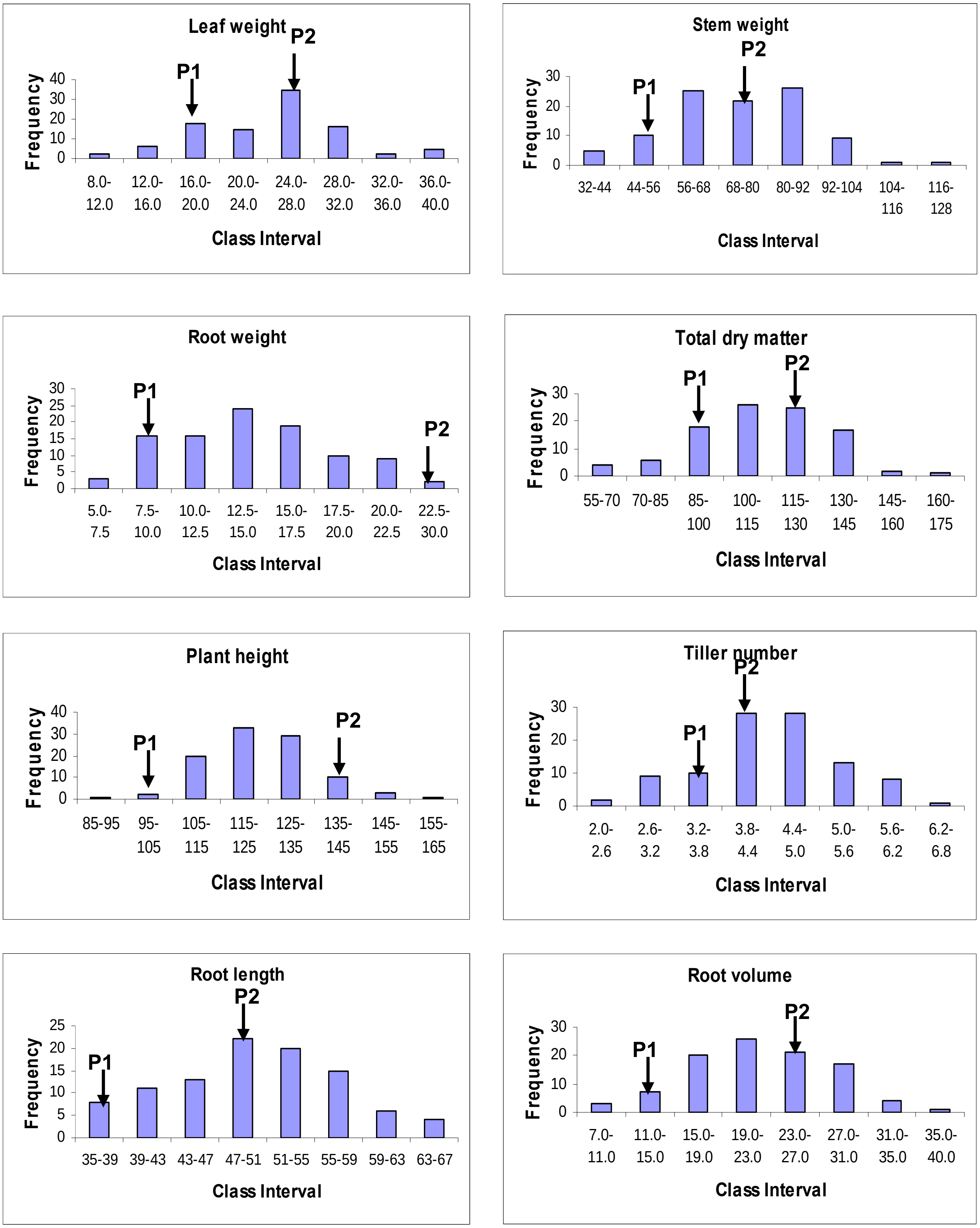
Two parents contrasting for drought and blast were phenotyped. IE 2912 performed well in many of the traits. Further RILs were developed from the cross between above mentioned parents

Table 30 : Genetic variability of physiological traits among recombinant inbred lines of the mapping population (IE 2912xIE 2885) in finger millet in second season

Trait	Minimum	Maximum	Mean	P value	SEM	$CD_{0.05}$	CV %	Kurtosis	Skewness
Leaf weight (g pl^{-1})	8.94	38.42	24.19	**	0.60	0.60	8.89	-0.05	0.04
Stem weight (g pl^{-1})	38.42	127.31	73.23	**	1.67	0.80	3.88	0.23	0.15
Plant height (cm)	90.22	157.23	124.37	**	1.24	1.08	3.44	0.05	0.20
Tiller number	2.11	6.21	4.48	**	0.08	0.73	2.08	-0.28	-0.11
Root length (cm)	35.05	66.41	50.25	**	0.76	0.09	7.66	-0.49	-0.06
Root volume (cm^3)	7.78	39.98	22.10	**	0.60	0.60	4.26	0.04	0.09
Root dry weight (g pl^{-1})	6.86	29.18	14.18	**	0.43	0.50	9.50	0.31	0.54
Root/Shoot	0.144	0.176	0.16	NS	0.004	0.05	14.56	4.31	1.35
Total dry matter (g pl^{-1})	60.15	168.72	111.61	**	2.22	0.45	11.37	-0.28	-0.15

****significant at 5 %**
NS-Non significant

Along with parents mapping population was also phenotyped, Most of the physiological traits in second season experiment varied significantly except root to shoot ratio.



P1-IE 2885
P2-IE 2912

Fig. 13 : Frequency distribution of physiological traits in the second season experiment

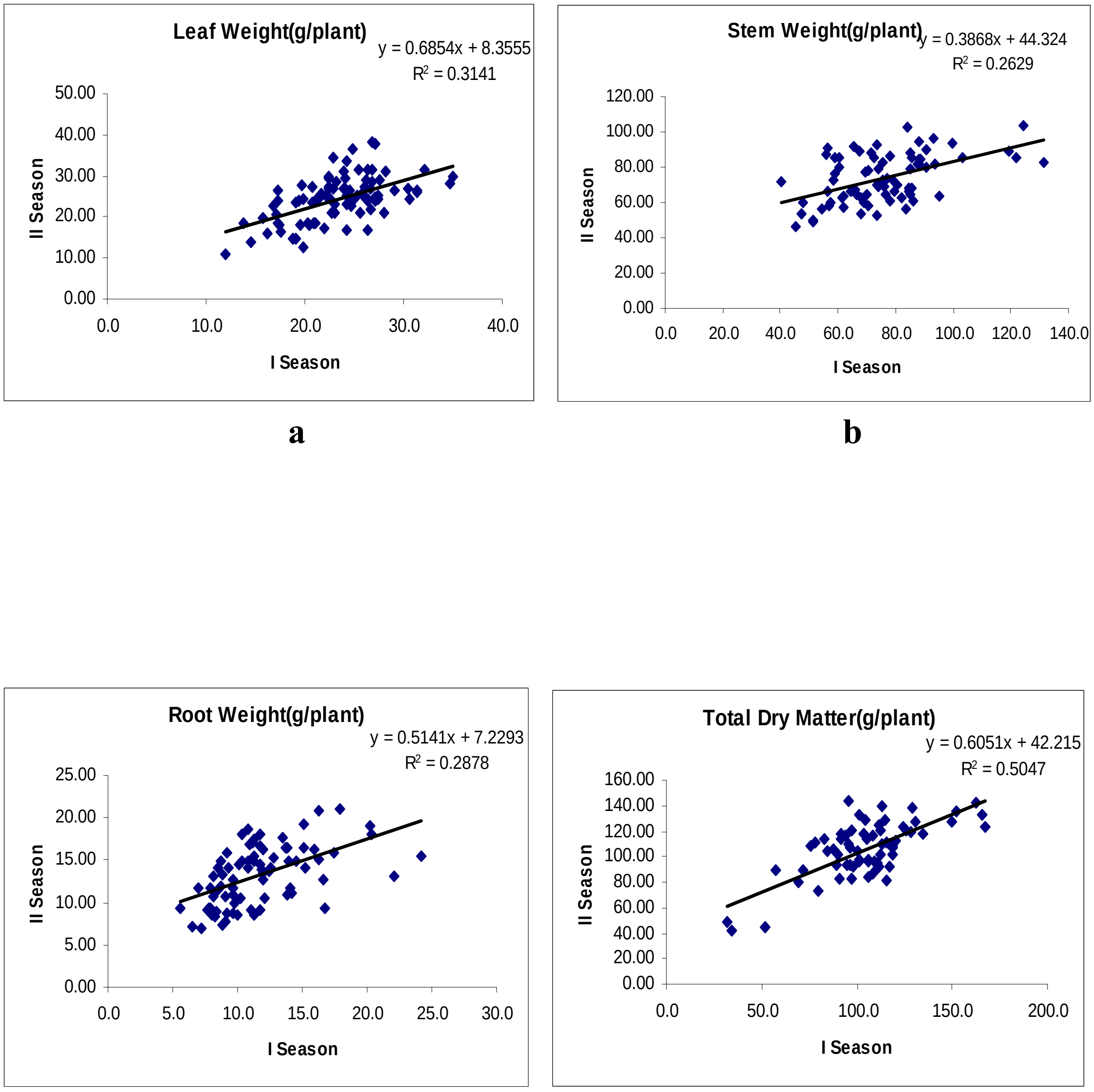
positively skewed indicating the predominance of transgressive segregants towards the superior parent.

Several parameters associated with shoot growth such as plant height, number of tillers and shoot weight were recorded. The tiller number varied significantly from 2.11 in MLC 17-3 to 6.21 in MLC 82-3. RIL MLC 74-3 showed the lowest stem weight of 38.42 g.plant^{-1} and RIL MLC 29-1 showed a highest stem weight of 127.31 g.plant^{-1}. The mean plant height of RILs was 124.37 cm ranging from 90.22 cm in MLC 9-4 to 157.23 cm in MLC 7-4 representing a significant variability. The shoot associated traits exhibited normal distribution as indicated in Fig 13. The stem weight (0.15) and plant height (0.20) were positively skewed whereas tiller number was negatively skewed (-0.11). Both stem weight (0.23) and plant height (0.05) revealed positive kurtosis whereas tiller number showed negative kurtosis (-0.28).

The RILs had a mean root length of 50.25 cm whereas the mean for root weight was 14.18 g plant^{-1}. The lowest root weight of 6.86 g plant^{-1} was noticed in RIL MLC 55-5-1 and the highest was noticed in MLC 15-2 (29.18 g plant^{-1}). No significant difference was observed in root to shoot ratio. The frequency distribution of root traits showed continuous variability confirming the polygenic inheritance of the trait. Except root length (-0.06) other root traits such as root volume (0.09), root weight (0.54) and root to shoot ratio (1.35) were positively skewed with high kurtosis indicating that majority of the recombinant inbred lines performed better than the superior parents for these traits. The kurtosis for root to shoot ratio (4.31) was maximum among the root traits. The BM in the population varied significantly around the mean of 111.61 g pl^{-1} ranging between 60.15 g pl^{-1} (MLC 74-3-1) and 168.72 g pl^{-1} in MLC 100-4 which is normally distributed with negative skewness (-0.15) and kurtosis (-0.28).

4.14 Stability of Physiological traits

Stability of a trait across seasons is essential for breeding programs to be successful. We assessed the stability for several physiological traits. Significant positive correlation was seen for number of growth parameters like total biomass, root characteristics and also tillering ability between two seasons (Fig 14 & 15).



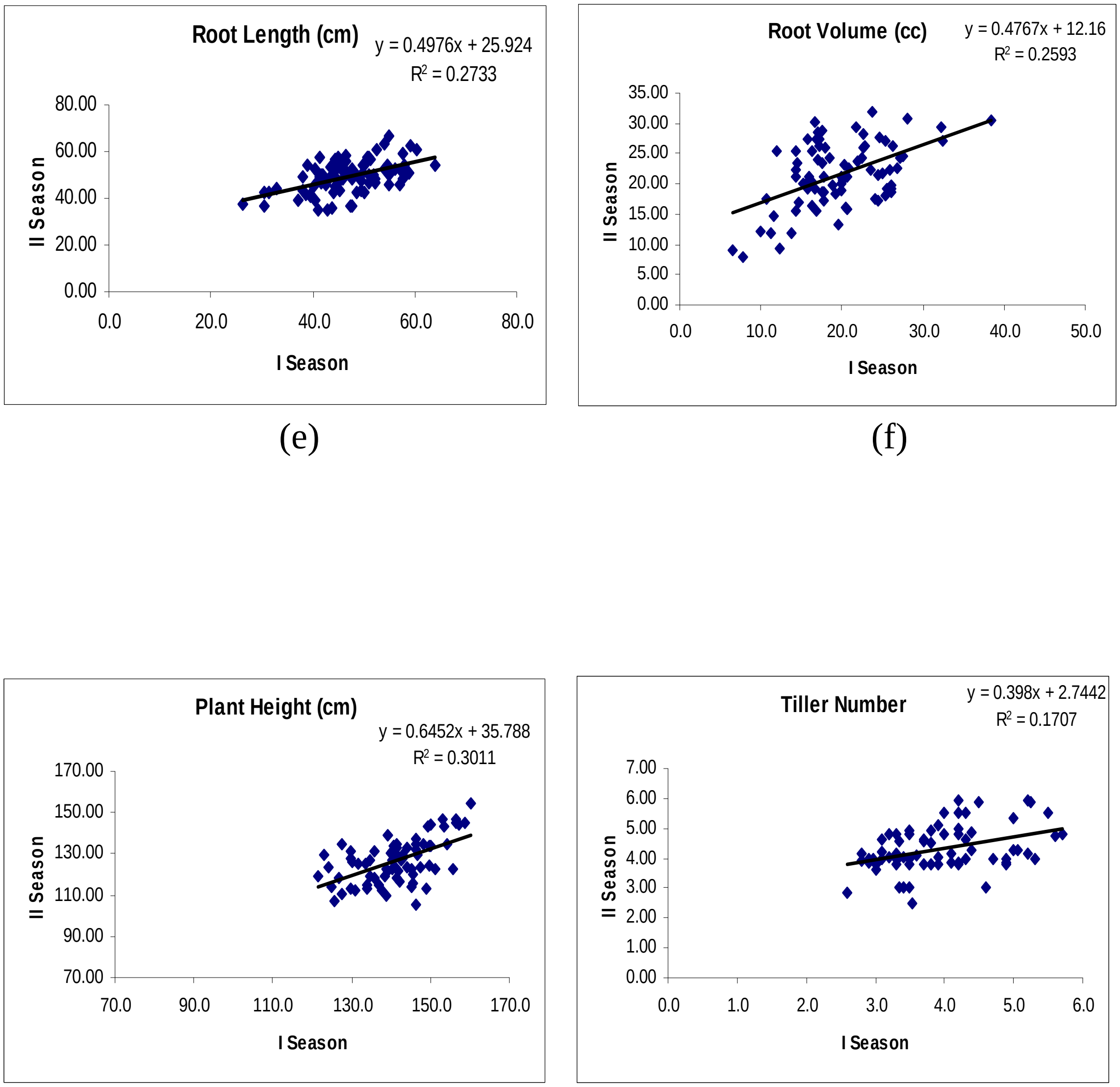
a

b

c

d

Fig. 14 : Stability of physiological traits (a) leaf weight, (b) stem weight, (c) root weight and (d) total dry matter between two seasons through correlation analysis



(e) (f)

(g) (h)

Fig. 15 : Stability of physiological traits (e) root length, (f) root volume, (g) plant height and (h) tiller number per plant between two seasons through correlation analysis.

4.15 Molecular characterization of RILs

The other major emphasis of this investigation was to exploit the genomic resources of SSR markers developed as well as those available in public domain for molecular characterization of the mapping population. Initially, genomic and genic SSR markers and RAPD markers were screened for parental polymorphism. Subsequently, the polymorphic markers were used to screen the mapping population. Following section describes the molecular characterization leading to the development of genetic linkage map and identification of QTL (quantitative trait loci).

4.15a Parental polymorphism using in-house developed SSR markers

In house developed genomic SSR primers were screened between the two parents (IE 2912 & IE 2885) (Plate8). Around ten SSR primers showed polymorphism out of 156 SSR primers which were initially standardized for their locus specific amplification (plate8a).

The parents were screened with outsourced Finger millet and Rice SSR primers. Five out of 31 standardized FM SSR primers revealed polymorphism between the parents (plate8b). Sixty nine rice SSR primers were standardized for their amplification in finger millet and also used for parental polymorphism analysis, but none of them revealed polymorphism between the parents. Apart from genomic and public domain SSRs, 87 genic/EST SSRs were also used for assessing the parental polymorphism, but none of them showed polymorphism between parents. The details of SSR primers used in the present study is mentioned in the Table 31

4.15b Genotyping: Screening of polymorphic SSR markers in RILs

The polymorphic SSRs were used for screening the recombinant inbred lines (RILs) (Plate9).

4.15c Parental polymorphism using RAPD markers

Along with the SSR markers, RAPD markers were also used in this study. Totally 315 RAPD primers were screened between the two parents (IE 2912 & IE 2885). Around

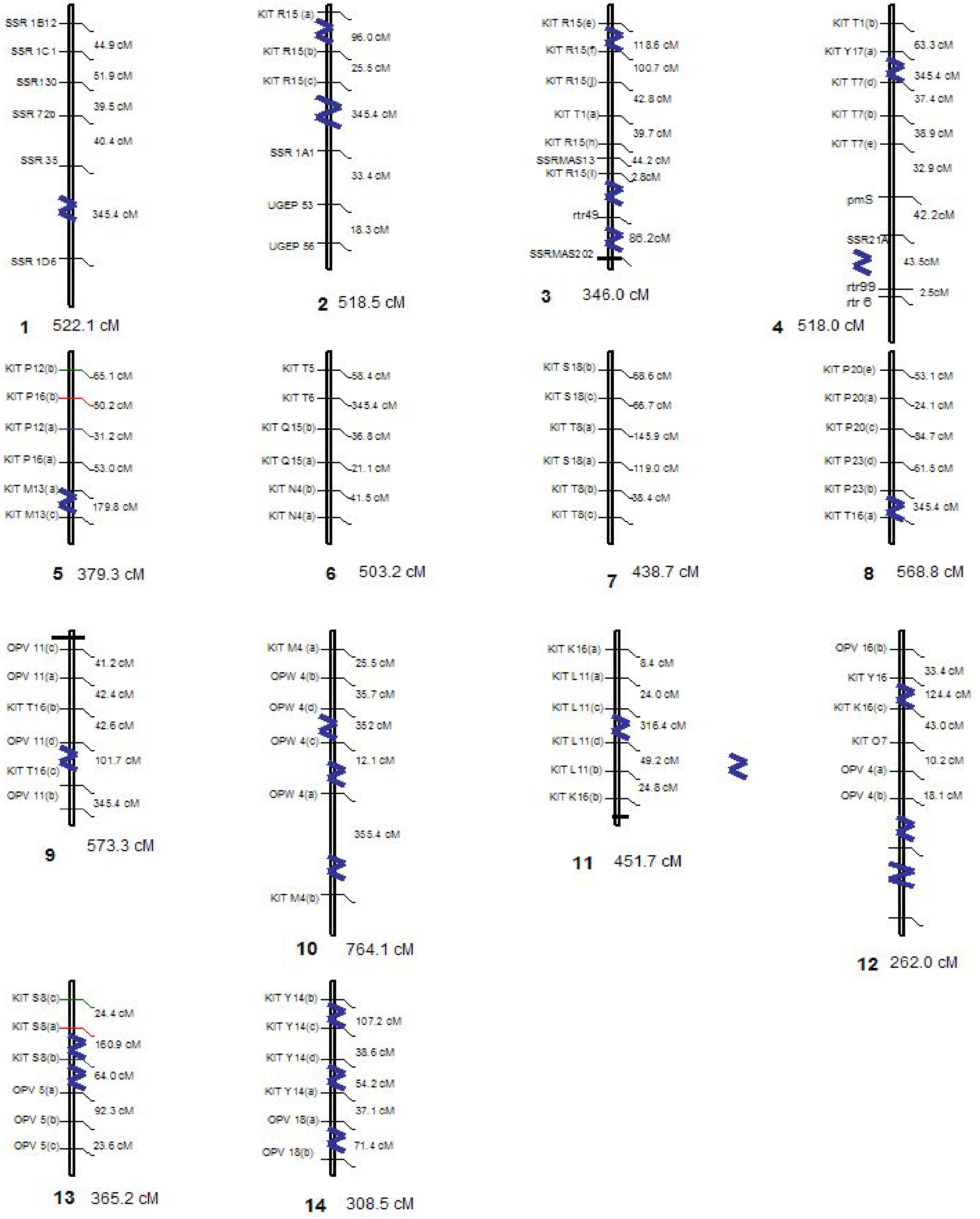


Fig. 16 : Genetic map of the finger millet genome

A skeletal linkage map was constructed with SSR and RAPD markers using 150 RILs derived from the cross between IE 2912 X IE2885. The map was constructed using the software MAPMAKER/EXP 3.0. The linkage groups were created with a LOD score of 3.0 and with a maximum distance threshold of 50cM which formed 14 linkage groups.

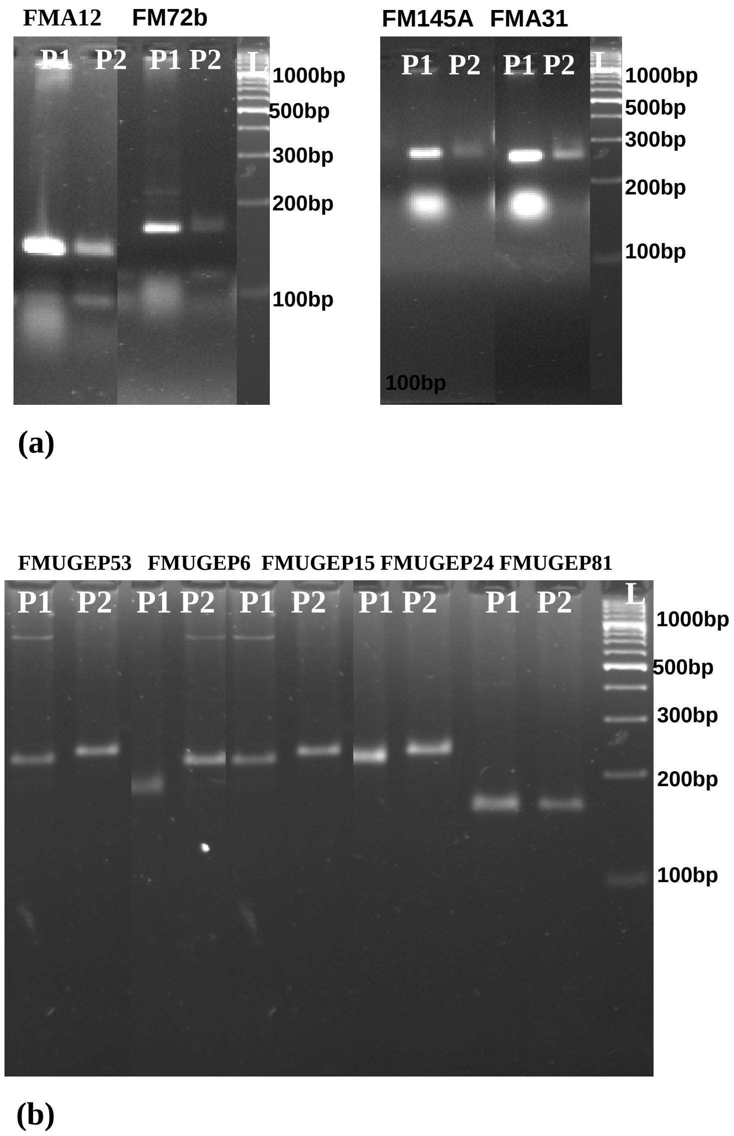


Plate 8 : Parental polymorphism analysis using in-house developed (a) and outsourced SSR primers (b)

Once the primers studied for their locus specific amplification, further such standardized SSRs(both in-house developed and outsourced) primers were screened in two parents (IE 2912 & IE 2885) to study polymorphism. Above shown primers revealed polymorphism with product size ranged from 150-300 bp.

P1-IE2912, P2-IE2885, L-Ladder

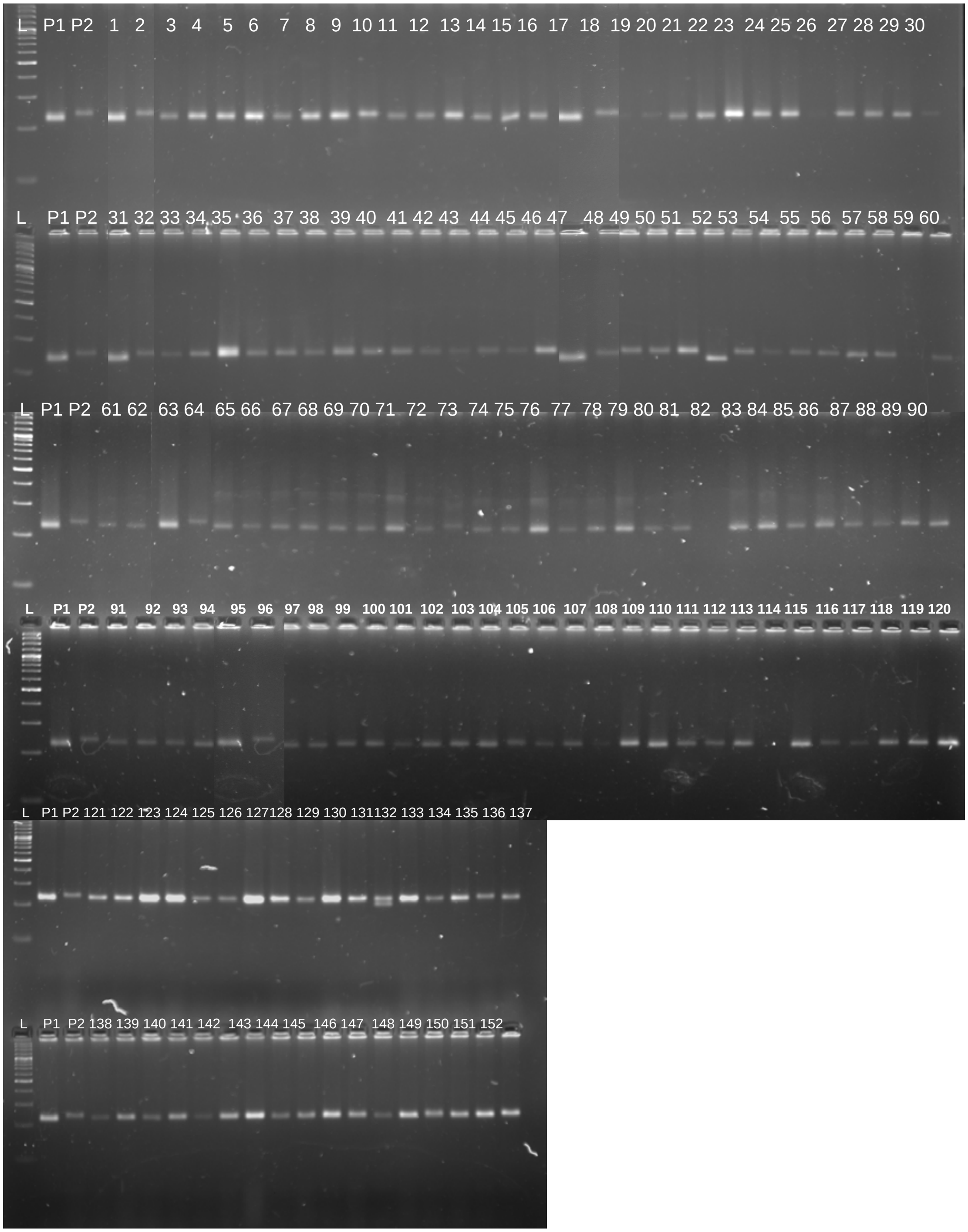


Plate 9 : SSR profile of the primer FMUGEP53 for the parents (IE2912xIE2885) and RILs

L-Ladder 100bp, P1-IE2912, P2-IE2885, other lanes indicate RILs

The polymorphic SSRs (both in-house developed and outsourced) were further genotyped in mapping population along with parental lines in finger millet.

32 primers revealed parental polymorphism (Plate10) contributing ninety five loci. The summary of the RAPD primers used in the study are shown in the table 32. Details RAPD primers used in the present study mentioned in table 33

4.15d Screening of polymorphic RAPD markers in RILs

Thirty two RAPD primers which revealed polymorphism in parents were further screened in RILs. Genotyping of RILs along with two contrasting parents using one of RAPD primers is shown in the Plate11

4.16 Construction of Linkage map using MAPMAKER/EXP 3.0

The polymorphic markers were run on the individuals of the RIL population in order to generate the marker data for linkage map construction. A skeletal linkage map was constructed with 95 RAPD and 15 SSR marker loci using 150 RILs. The map was constructed using the software MAPMAKER/EXP 3.0 (by Dr Shailaja Hittalamani, Professor and Head Department of Genetics & Plant Breeding). The linkage groups were created with a LOD score of 3.0 and with a maximum distance threshold of 50cM which formed 14 linkage groups. A minimum of 4 markers mapped on the linkage group 14 and a maximum of 9 markers covered the linkage groups 3 and 4. In total, 102 markers covering 6519.4 cM on 14 linkage groups were mapped. The marker names are represented on the left -hand side of the linkage group, whereas the marker positions in terms of centimorgan (cM) are represented on the right hand side (Fig.16).

4.17 Association of Markers/QTLs for physiological traits

The results of the genetic polymorphism were utilized for the construction of genetic linkage map as well as to associate markers. The marker association to a trait was performed by Single Marker Analysis (SMA). .

4.17a Single Marker Analysis (SMA)

The linear regression analysis was performed between the marker loci and the relevant physiological traits. The MS EXCEL software was used to run this analysis to associate the marker with the traits at a probability level of < 0.05.

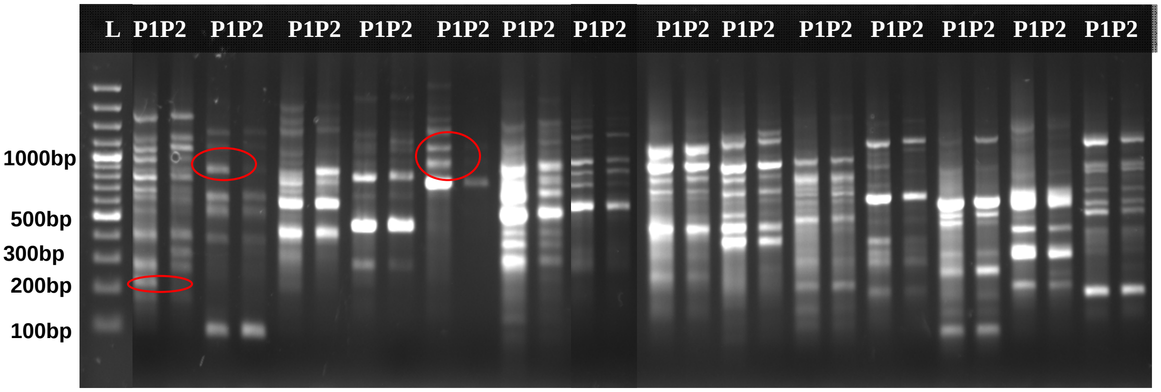


Plate 10 : Parental polymorphism analysis using RAPD primers

In addition to SSR primers, RAPD primers were also used in the present study for parental polymorphism which further used in genotyping.

P1-IE2912, P2-IE2885, L-Ladder

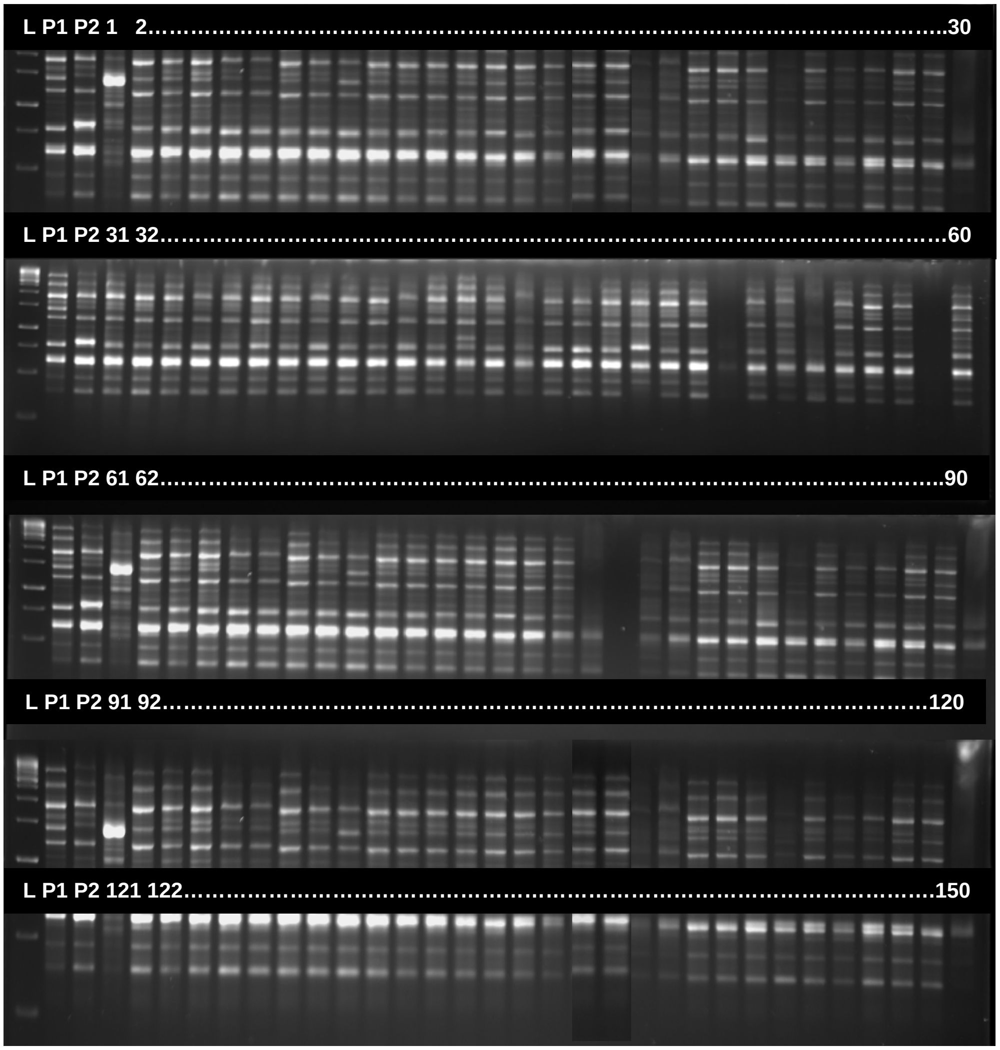


Plate 11 : RAPD profile of the primer KIT T16 for the parents (IE2912xIE2885) and RILs

Initially large number of RAPD primers analysed for parental polymorphism, further The polymorphic RAPD primers were used for genotyping in mapping population

L-Ladder 100bp, P1-IE2912, P2-IE2885, other lanes indicate RILs

Table 31 : Summary of the SSR primers used in the study

Sl. No.	Type of SSR marker	No. of primers analyzed	Locus specific amplification	Polymorphic SSRs
	Genomic SSRs	323	156	10
	Public domain SSRs			
	Rice SSRs	228	69	-
	Finger millet SSRs	31	31	5
	Genic SSRs	149	87	-
	Total	**731**	343	**15**

In the presIn the present study, both in house developed (genomic SSRs) and outsourced (rice and finger millet SSRs) along with genic SSRs were analyzed for their locus specific amplification. The standardized primers were used for parental polymorphism.

Table 32 : Summary of the RAPD primers used in the study

No. of RAPD primers screened	No. of Polymorphic RAPD primers	No. of Polymorphic loci
315	32	95

In addition to in house developed and outsourced marker systems, initial screening of large number of RAPD markers was also done in the present study. Further the standardized RAPD primers used for parental polymorphic analysis.

Table 33 : Details of RAPD primers used in the present study

S.N	RAPD Primers	Remarks	No. of polymorphic loci
1	OPV4	Polymorphic	2
2	OPV5	Polymorphic	3
3	OPV8	Monomorphic	
4	OPV11	Polymorphic	4
5	OPV16	Polymorphic	2
6	OPV17	Monomorphic	
7	OPV18	Polymorphic	2
8	OPV19	Monomorphic	
9	OPV20	Monomorphic	
10	OPW1	Monomorphic	
11	OPW4	Polymorphic	4
12	OPW7	Monomorphic	
13	OPW11	Monomorphic	
14	OPW20	Monomorphic	
15	KIT X6	Monomorphic	
16	KIT X12	Monomorphic	
17	KIT X18	Monomorphic	
18	KIT Y4	Monomorphic	
19	KIT Y6	Monomorphic	
20	KIT Y7	Monomorphic	
21	KIT Y11	Polymorphic	2
22	KIT Y13	Monomorphic	
23	KIT Y14	Polymorphic	4
24	KIT Y16	Polymorphic	1
25	KIT Y17	Polymorphic	2
26	KIT Y18	Polymorphic	4
27	KIT Y19	Monomorphic	
28	KIT Z5	Monomorphic	
29	KIT Z7	Monomorphic	
30	KIT Z11	Monomorphic	
31	KIT Z20	Monomorphic	
32	KIT K11	Monomorphic	
33	KIT K12	Polymorphic	1
34	KIT K14	Monomorphic	
35	KIT K16	Polymorphic	3
36	KIT L2	Monomorphic	
37	KIT L3	Monomorphic	

Continued..

38	KIT L4	Monomorphic	
39	KIT L8	Monomorphic	
40	KIT L11	Polymorphic	4
41	KIT L12	Monomorphic	
42	KIT L14	Monomorphic	
43	KIT L17	Monomorphic	
44	KIT L18	Monomorphic	
45	KIT M4	Polymorphic	2
46	KIT M5	Polymorphic	2
47	KIT M6	Monomorphic	
48	KIT M8	Monomorphic	
49	KIT M13	Polymorphic	3
50	KIT N4	Polymorphic	4
51	KIT N10	Monomorphic	
52	KIT N11	Monomorphic	
53	KIT N14	Monomorphic	
54	KIT N16	Monomorphic	
55	KIT O4	Monomorphic	
56	KIT O6	Monomorphic	
57	KIT O7	Polymorphic	1
58	KIT O11	Monomorphic	
59	KIT O12	Monomorphic	
60	KIT O13	Monomorphic	
61	KIT O15	Monomorphic	
62	KIT O16	Monomorphic	
63	KIT S1	Monomorphic	
64	KIT S8	Polymorphic	3
65	KIT S18	Polymorphic	5
66	KIT S20	Monomorphic	
67	KIT P1	Monomorphic	
68	KIT P2	Monomorphic	
69	KIT P3	Monomorphic	
70	KIT P12	Polymorphic	2
71	KIT P16	Polymorphic	3
72	KIT P20	Polymorphic	5
73	KIT Q12	Monomorphic	
74	KIT Q14	Monomorphic	
75	KIT Q15	Polymorphic	2
76	KIT R15	Polymorphic	10
77	KIT R20	Monomorphic	
78	KIT T1	Polymorphic	2
79	KIT T5	Polymorphic	1
80	KIT T6	Polymorphic	1
81	KIT T7	Polymorphic	5
82	KIT T8	Polymorphic	3
83	KIT T15	Monomorphic	
84	KIT T16	Polymorphic	3

4.17b Association of Markers for Leaf and Shoot traits

The markers associated with leaf traits are presented in Table 34. A total of six markers were associated to specific leaf area. RAPD marker KITS8c explained maximum phenotypic variation of 8.5% (p=0.0002), whereas the SSR marker SSR1C1 explained the minimum phenotypic variation of 2.68% at p= 0.044. Eleven markers were found to be associated with total leaf weight, explaining about 2.67% to 6.52% of the phenotypic variation by KITP20e (p=0.044) and KITR15b (p=0.001) respectively. Eight markers were associated with total leaf area. Among them, RAPD marker OPV11c explained maximum phenotypic variance of 6.52 (p=0.001), whereas minimum phenotypic variance was explained by SSR marker SSR61 i.e., 2.53% (0.050).Among the markers associated to shoot weight, KITS8c showed a maximum phenotypic variation of 4.94% (p = 0.006) and KITY14a showed a minimum phenotypic variation of 3.12% (p = 0.029) (Table 35). A total of about seven markers were associated with plant height, among them RAPD marker KIT11a explained maximum phenotypic variance of 4.95 (p=0.005) whereas minimum phenotypic variance was explained by RAPD marker OPW4b i.e., 2.52% (p=0.051). Fourteen markers were associated with tiller number. The markers associated with tiller number explained a phenotypic variation ranging from 2.45% (OPV16b) to 7.76% (KITR15g).

4.17c Association of Markers for Root traits

The markers associated with root traits are presented in Table36. A total of ten markers were associated to root length. RAPD marker KIT11a explained maximum phenotypic variation of 10.75% (p=0.0001), whereas the RAPD marker KITT8a explained the minimum phenotypic variation of 3.07% at (p= 0.031). Thirteen markers were found to be associated with total root volume, explaining about 2.45% to 6.08% of the phenotypic variation by SSR marker FMUGEP53 (p=0.054) and RAPD marker KITP20d (p=0.002) respectively. Eight markers were associated with root weight. Among them, RAPD marker KITP20e explained maximum phenotypic variance of 6.54 (p=0.001), whereas minimum phenotypic variance was explained by RAPD marker KITN4a i.e., 2.70% (0.043). The markers associated with root length and root weight are given. A total of 10 markers were associated to root length. RAPD marker KIT11a

Table 34 : Markers associated with leaf traits

Trait	Marker	R^2	'P' Value
Specific Leaf Area	KITP20c	3.02	0.032
	KITS8a	3.72	0.017
	KITS8c	8.50	0.0002
	KITT16b	2.85	0.037
	SSR 1C1	2.68	0.044
	SSR35	2.75	0.040
Leaf Weight	KITP20e	2.67	0.044
	KITR15b	6.52	0.001
	KITR15g	3.53	0.020
	KITT1b	3.33	0.024
	KITS8a	4.88	0.006
	KITT16C	2.91	0.035
	KITY14a	4.75	0.007
	KITY14c	3.88	0.015
	OPV5b	4.11	0.012
	OPV5c	4.26	0.010
	OPV16b	3.69	0.017
Total Leaf Area	SSR35	5.08	0.005
	SSR61	2.53	0.050
	UGEP24	3.32	0.024
	KITN4c	2.85	0.038
	KITT6	2.58	0.048
	OPV11c	6.52	0.001
	KITL11d	4.11	0.012
	KITK16b	2.85	0.038

To know the association of marker and physiological trait Single marker analysis was performed. Leaf traits such as SLA, LW and TLA associated with 6, 11 and 8 markers respectively.

R^2= Percent variation explained
P= Probability

Table 35 : Markers associated with shoot traits

Trait	Marker	R^2	'P' Value
Stem Weight	KITS8c	4.94	0.006
	KITY14a	3.12	0.029
Plant Height	KITL11a	4.95	0.005
	KITO7	2.58	0.048
	KITP20b	4.1	0.012
	KITR15j	3.79	0.016
	KITT5	2.63	0.046
	KITY14c	4.19	0.011
	OPW4b	2.52	0.051
Tiller Number	KITK16a	3.12	0.029
	KITM13c	2.76	0.041
	KITN4a	2.76	0.041
	KITP16a	2.92	0.035
	KITP20e	2.68	0.044
	KITR15b	4.79	0.006
	KITR15c	6.21	0.002
	KITR15f	2.68	0.044
	KITR15g	7.76	0.000
	KITT7e	6.07	0.002
	KITT16C	3.03	0.032
	KITY14a	2.63	0.046
	KITY17a	3.92	0.014
	OPV16b	2.45	0.054

Singer marker analysis revealed that shoot traits such as tiller number and plant height associated with 14 and 7 markers respectively where as stem weight was associated with only two markers.

R^2= Percent variation explained
P= Probability

Table 36 : Markers associated with root traits

Trait	Marker	R^2	‘P’ Value
Root Length	KITL11a	10.75	0.0005
	KITP20d	3.98	0.014
	KITT7e	3.46	0.022
	KITT8a	3.07	0.031
	KITT8b	3.48	0.021
	KITY17a	6.34	0.001
	OPV16b	3.26	0.026
	OPW4a	3.40	0.023
	SSR1D6	6.40	0.001
	SSR35	3.36	0.024
Root Volume	FMUGEP53	2.45	0.054
	KITM13c	5.25	0.004
	KITP12a	2.94	0.035
	KITP20d	6.08	0.002
	KITP20e	2.58	0.048
	KITR15c	3.03	0.032
	KITR15f	2.85	0.038
	KITR15g	3.15	0.029
	KITT7b	3.21	0.027
	KITT16C	3.27	0.026
	KITY17a	4.29	0.010
	SSR1B12	4.01	0.013
	SSR1D6	3.01	0.032
Root Weight	KITN4a	2.7	0.043
	KITP20e	6.54	0.001
	KITR15b	5.38	0.004
	KITR15g	4.07	0.012
	KITS8a	4.64	0.007
	KITS8c	5.08	0.005
	KITT8a	3.39	0.023
	KITT16C	3.02	0.032
	OPV5a	4.03	0.013
	OPW4b	3.14	0.029
	OPW4b	3.14	0.029
Root Shoot Ratio	FMUGEP53	11.1	0.0001
	KIT11b	7.99	0.0004
	KITP20c	2.94	0.035
	OPV4a	3.95	0.014
	OPW4b	3.18	0.027

R^2= Percent variation explained P= Probability explained maximum phenotypic variation of 10.75% (p=0.0005), whereas the RAPD marker KITT8a explained the minimum phenotypic variation of 3.07% at p= 0.044. Similarly, 11 markers were found to be associated with root weight, explaining 2.7% to 6.54% of phenotypic variation. Markers associated with root volume explained a variation ranging from 2.45% (FMUGEP53) to 6.08 % (KITP20d). For root to shoot trait 5 markers were found to be associated wherein SSR marker FMUGEP53 explained maximum phenotypic variation of 11.1% where as minimum phenotypic variation of 2.94 explained by RAPD marker KITP20c. Eight markers found to be associated with biomass (Table 37) with phenotypic variance ranging from 2.53% (RAPD marker KITY14c) to 4.91% (RAPD marker KITS8c).

Table 37 : Markers associated with biomass

Trait	**Marker**	**R^2**	**'P' Value**
BM	KITN4a	3.03	0.032
	KITP20e	2.56	0.049
	KITR15b	2.57	0.049
	KITR15g	3.24	0.026
	KITS8c	4.91	0.006
	KITT8a	3.24	0.026
	KITY14a	3.98	0.014
	KITY14c	2.53	0.050

Eight markers found to be associated with biomass with phenotypic variance ranging from 2.53% to 4.91%

R^2= Percent variation explained P= Probability

Discussion

V DISCUSSION

Finger millet accounts for 8% of the area and 11% of production of all millets in the world (Bennetzen *et al.*, 2003). Finger millet is grown on over 4 million ha world wide and it is a primary food for millions in dry lands of East and Central Africa and Southern India. This crop is grown under both irrigated and dry land conditions in southern parts of India. Karnataka alone accounts for 2.6 million hectares, producing around 3 million tons. Unlike major millets (sorghum and pearl millet), finger millet is a coarse millet having relatively less deep root system leading to poor water mining from the deeper layers of soil. In this scenario, physiological characterization and molecular breeding for root traits in finger millet becomes crucial. Hence there is a large scope to improve the root system in finger millet.

Drought is a complex abiotic environmental factor that causes water deficit or water stress in plants. One of the challenges in crop improvement is to achieve good productivity under drought conditions. Assessing drought itself is difficult task as it is a complex process and drought traits are multigenic controlled by many genes. Internal water deficit in plants is initiated when water potential decreases and cell turgor begins to fall below its maximum value (Kozlowski and Pallardy, 1997).

Efforts to increase water harvesting and its efficient utilization has been the main focus of interest in the recent years. Dehydration avoidance is one of the adaption mechanisms, through which plants survive and yield better under stress conditions. Therefore deep root system is known to be one of the best strategies to cope with the stressful situations (Ingram *et al.*, 1994). In fact enhanced water uptake by efficient root characteristics is a desirable physiological mechanism of dehydration avoidance under limiting conditions. Hence, identifying genotypes with desirable root characteristics for moisture stress condition would be very useful. (Kramer and Boyer, 1995; Turner, 1997). However knowledge of root traits associated with drought tolerance is important for further understanding drought tolerance mechanisms of the whole plant.

Furthermore, particularly in the tropics, drought spells are coupled with both high solar radiation and temperature remarkably aggravating (DaMatta, 2003) the water stress.

Therefore, thorough analysis of the physiological events during drought stress and their genetic control is essential to define regulatory genes, which are primary and positively contributing to stress tolerance.

Any drought tolerant trait would be relevant for crop improvement only when it is associated with crop growth rate (Udayakumar and Prasad, 1994). From this perspective, a high ratio of carbon gained per unit water lost through transpiration, namely high water use efficiency (WUE), is hypothesized to confirm a fitness advantage under drought stress, (Cohen, 1970; Dudley, 1996; McKay *et al.*, 2001). Understanding the genetic basis of WUE is therefore important to improve crop productivity under water-limited conditions.

The combination of relevant physiological traits must be pyramided in order to achieve an overall improvement in drought tolerance as well as enhanced productivity under such conditions. However, breeding for these physiological traits is complex as these traits have polygenic inheritance and are also difficult to quantify. Thus, molecular breeding strategy using tightly linked DNA based molecular markers to identify the desirable genotype is expected to significantly complement the breeding processes to introgress relevant traits.

Quantitative trait loci (QTL) mapping is a complementary approach useful for identifying the genes underlying naturally occurring variation in traits such as those conferring drought adaptation. QTL mapping allows one to statistically identify chromosomal regions containing genetic factors contributing to variation in a polygenic trait (Lynch & Walsh, 1998; Alonso-Blanco & Koornneef, 2000). Once the tightly linked markers have been identified, they can be used to develop marker assisted selection strategy for breeding application. Molecular markers allow breeders to track genetic loci controlling drought resistance without having to measure the phenotype, thus reducing the need for extensive field-testing over space and time

To achieve this, the prerequisites are

- Development of trait specific mapping population with contrasting parents
- Efficient marker systems
- Accurate Phenotyping of mapping population and contrasting parents

In finger millet, some physiological traits have been shown to potentially contribute to yield under water stress conditions but the development of an efficient breeding method for drought tolerance is still a long-standing objective. In view of the time-consuming process in finger millet breeding, modern molecular technologies like DNA markers have a great potential to assist in and accelerate breeding programmes. But lack of good co-dominant markers (such as SSRs) is one of the reasons for slow progress in breeding activities of this crop.

The results of the present investigation on development of SSR markers and identification & validation of markers linked to root and associated physiological traits in finger millet have been discussed in this chapter.

Development of genomic SSR markers in finger millet

Success of molecular characterization depends on the availability of locus specific markers. In spite of the fact that diverse markers are being used like RAPD, SSR etc., emphasis lies on the co-dominant SSRs, SNPs and new generation markers. In crops where there are adequate markers like rice and maize, there is phenomenal progress in marker assisted selection. Therefore the primary limitation here is to generate marker systems. In finger millet there are hardly any locus specific markers.

Simple sequence repeats (SSRs) are highly polymorphic repeated sequences which are multiallelic molecular markers, because they are co-dominant and enable the heterozygous samples to be distinguished from the homozygous situation. In addition they are simple, PCR based and highly informative due to number and frequency of alleles detected and to their ability to distinguish between closely related individuals. Due to these characteristics, SSRs are powerful tools for tracing specific genes in crossing programmes. They find application as markers for mapping, cultivar identification, protecting germplasm, hybrid determination, analysis of gene pool variation and as diagnostic markers for traits of economic value (Powell *et al.*, 1996). Such genetic markers enable to track the inheritance of a particular marker allele and a measured quantitative trait provides evidence that QTL is linked to marker. Therefore molecular marker linkage maps are being used successfully in associating the traits.

Different approaches for microsatellite isolation

There are different approaches for microsatellite isolation from plant genome like traditional, primer extension and selective hybridization (pre-cloning enrichment) methods.

Traditionally, microsatellite loci have been isolated from partial genomic libraries (selected for small insert size) of the species of interest, screening several thousands of clones through colony hybridization with repeat containing probes (Rassmann *et al.*, 1991). Although traditional method is relatively simple, especially for microsatellite rich genomes, this approach can turn out to be extremely tedious and inefficient for species with low microsatellite frequencies and also more time consuming with low yield. Another method for microsatellite isolation i.e., based on primer extension, has been proposed for the production of libraries enriched in microsatellite loci for efficient enrichment of AC repeats yielding from 40-50 % (Ostrander *et al.*,1992) upto 100% positive clones (Paetkau,1999). These protocols involve high number of steps which might explain their limited application. Many of these experiments concerned the isolation of dinucleotide repeat microsatellites, and it is unclear whether the primer-extension approach is effective also for tri- and tetranucleotide.

The best alternative to both traditional and primer extension methods is enrichment or selective hybridization strategy which is performed using an oligonucleotide containing several tandem repeats of the motif to be enriched as a probe. The probe can be cross linked to a nylon membrane or can be biotinylated at the 5’ end, so that DNA hybridized with the probe can be selectively removed using streptavidin-coated paramagnetic beads. This enrichment strategy is quick, efficient in isolating different kinds of repeats with good yield.

In the present study, for the first time an attempt was made to isolate microsatellites in fingermillet using pre-cloning enrichment strategy which resulted in isolation of very good number of micro satellites (323). While developing the microsatellite-enriched library for finger millet, more than 2200 colonies were screened, 1021 recombinant clones ranging from 500-1000bp were selected for further sequencing. Sequence analysis revealed that only 645 clones (63%) contained microsatellite motif regions, while many traditional microsatellite isolation studies have reported an average of 50-60% positive clones (Zane *et al.,* 2002) after sequencing and the average per cent of false positives in such studies was estimated to be 48.7% (Squirrel *et al.,* 2003). The short length of oligonucleotides used as probes could be a possible reason for the resulting high proportion of false positives. The length of the probe applied for hybridization can influence the type and length of microsatellites captured. Armour *et al* (1994) found that longer oligonucleotide probes not only favored the isolation of relatively long arrays, but also eliminated the mismatches. While the protocol in this study made use of (28-30 bp long) oligonucleotides, other more successful studies (Stajner *et al*, 2005) used much longer probes (200-550 bp). It has been suggested that short probes for hybridization tend to select fragments with shorter interrupted repeats. In other investigations (Lopes *et al.,* 2002; Senda *et al.,* 2004), 2 rounds of hybridization have been performed to eliminate false positives. A second hybridization is especially recommended, if inefficient labeling of oligonucleotide probes (by using incorrect concentration of probes, any form of contamination, or inadequate hybridization and wash steps) was suspected in the first round of screening. In pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Budak *et al.,* 2003) for example, a second round of hybridization managed to discard more than 50% of clones (46 out of 80) as false positives.

Several authors (Edwards *et al.,* 1996; Panaud *et al.,* 1996) have suggested enrichment of genomic libraries to enhance identification of microsatellites. Enrichment ratios published in the literature vary from 20-95% depending mainly on the procedure and method used (Stajner *et al.,* 2005). In fact, the enriched library prepared in this study resulted in a higher percentage (63%) of microsatellites.

Characteristics of isolated SSR markers

In the present study, monorepeats C and T were abundant. AG and CT class of dinucleotide repeats accounted about 21% and 30% respectively (Table 9) and this is consistent with the previous studies in fingermillet (Dida *et al.*, 2007), and also in Peach (Cipriani *et al.*, 1999) *Coffea* genus (Poncet *et al.,* 2006), hop (*Humulus lupulus* L.) (Stajner *et al.,* 2005), white clover (*Trifolium repens* L.) (Kölliker *et al.,* 2001) and *Lolium temulentum* (Senda *et al.,* 2004), for all of which enrichment strategy was employed further supporting the conclusion that these repeats are abundant in plants. GC repeats were not found, which is consistent with the reports suggesting that these repeats are extremely rare in most genomes (Cui *et al.,* 2005). Lower frequencies of GC repeats have been attributed to methylation of cytosine, which in turn increases the chance of mutation to thymine, by deamination (Schorderet and Gartlar, 1992).

A fair number of tri-nucleotide repeats (with more of TGA, TCA and GAT) were identified in the enrichment library (Table 10) which also corroborates with the results of Santos and his associates in 2003 in the enriched library of papaya.

Apart from the abundant, mono, di-nucleotide and tri repeats, about 58 tetranucleotide and 62 penta repeats were also isolated, with less success. Gaitán-Solis *et al.,* (2002) suggested that this failure could be due to selective enrichment of specific microsatellite sequences. However, the high success in enrichment of similar motifs in a separate study using similar enrichment (Métais *et al.,* 2002) strategy invalidates this point.

Although di-nucleotide repeats are the most commonly used class of microsatellite markers in plants, there has been concern over the difficulty experienced in genotyping them due to a high frequency of strand slippage artifacts (Levinson and Gutman 1987). In contrast, tri- and tetra-nucleotide repeat based markers have been shown to produce a higher proportion of discrete PCR products due to reduced level of stuttering (Gastier *et al.,* 1995). In human, these motifs have been found in genes that are associated with several neurological diseases such as X syndrome (Jin and Warren 2000), Huntingdon's disease and several forms of ataxia (Sermon *et al.,* 2001) and myotonic dystrophy (Timchenko *et al.,* 2001) which are caused by excessive expansions of triplet repeats located near or within coding regions (Fujimori *et al.,* 2003).

Locus specific amplification of isolated SSR markers

The sequencing was done in both the directions in order to increase the quality of sequences for primer designing after alignment. About 323 primers were designed out of which, 175 primers showed locus amplification. Few primers could not be optimized and they either produced faint bands or did not show reproducible banding patterns. Most of primers yielded sharp bands at a annealing of 55 ± 5^0C and, the bands were not sharp at 60^0C and often characterized with a smear or spurious bands presumably due to the high stringent annealing temperature. Although Squirrel *et al*, (2003) have indicated that optimization in itself could be a source of attrition and other investigations have pointed out the fact that PCR optimization may lead to insignificant improvement in band interpretability (Ashworth *et al.,* 2004). Out of 175 primers which showed locus specificity in the present study, very less number (10) of SSR markers revealed polymorphism between parents and also in mapping population. The low level of polymorphism can be attributed to genetic divergence of species.

Finger millet exhibits tetrasomic inheritance since it has a tetrploid genome. In such conditions, much more information is available if multiallelic markers such as microsatellites can be scored, as these facilitate identification of homologous chromosomes in both parents. Estimates of recombination frequencies based on multiallelic markers are upto four times as informative as the best estimates from dominant markers (Luo *et al.*, 2001).

Genic/EST SSRs

In recent years, large collections of ESTs that are available in some species have provided an alternative source of SSRs. EST-SSRs generally display lower levels of variation compared to genomic SSRs, but tend to have higher transferability across species (La Rota *et al.*, 2005). A higher level of polymorphism was detected in sorghum from sorghum genomic SSR markers than from sorghum EST-SSR markers. This result was consistent with a higher level of polymorphism detected from genomic SSRs than EST-derived SSRs in rice and durum wheat (Cho *et al.*, 2000; Eujayl *et al.*, 2002).

In the present study, around 796 stress specific ESTs clones were annotated for the presence of microsatellite repeats, genic SSR enriched clones, consisted more of monorepeats than direpeats which is contradictory to genomic enrichment , very few tri,tetra and penta repeats were found. Totally 149 genic/EST SSR primers, of which 92 primers were standardized for locus specificity but unable to reveal the polymorphism. Synteny studies with Rice EST SSR primers in fingermillet also showed amplification but did not reveal polymorphism.

Physiological characterization of mapping population

Although significant success was achieved in breeding for yield improvement, most of those efforts were based on selection for yield *per se*. This approach, however, is encountering increasing difficulties in achieving further improvement. A narrow variability in yield among the already improved cultivars, a large G X E interaction for yield have often been quoted as the reasons for slow progress in breeding for yield (Araus *et al.,* 2002; Richards *et al.,* 2002). To achieve further breakthrough in productivity, it is opined that the constituent physiological or morphological traits need to be improved. So the challenge of drought breeding can be addressed through “Trait based breeding approaches”.

Most of the physiological traits are quantitatively inherited and bringing together several such traits through conventional breeding would be a formidable task to achieve. Hence the concept of trait-based breeding would become relevant and achievable, only when robust selection techniques are evolved. One of the most powerful techniques for identifying these desirable individuals from breeding population is DNA based molecular marker. However, before attempting the marker-assisted selection, it is essential to first identify tightly linked DNA markers, with the traits of our interest.

To achieve the envisaged objectives of identifying DNA markers associated with the traits, accurate phenotyping in a suitable mapping population is one of the basic requirements. The construction of a linkage map requires a segregating plant population (i.e. a population derived from sexual reproduction). The parents selected for the mapping population will differ for one or more traits of interest. Population sizes used in preliminary genetic mapping studies generally range from 50 to 250 individuals (Mohan *et al.*, 1997), however larger populations are required for high-resolution mapping. Generally in self-pollinating species, mapping populations originate from parents that are both highly homozygous (inbred). In cross pollinating species, the situation is more complicated since most of these species do not tolerate inbreeding. Many cross pollinating plant species are also polyploid (contain several sets of chromosome pairs).

Mapping populations used for mapping cross pollinating species may be derived from a cross between a heterozygous parent and a haploid or homozygous parent (Wu *et al.*, 1992). Although mapping populations such as F2 populations and backcross populations are simple and takes less time to construct, but RILs and DH populations are homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring. This allows for the conduct of replicated trials across different locations and years indicating their relevance as ideal mapping population.

Thus a mapping population consisting of RILs derived from cross between IE 2912 and IE 2885 was used in the present study, which was basically developed for blast and drought associated traits. These two parents differed significantly in many traits such as biomass, leaf area, leaf weight, stem weight, plant height, root weight and root volume, but were nonsignificant in Δ^{13}C (Table 20). The same trend was also noticed in mapping population (Table 21). Similar results were reported by O'Leary (1988) in C4 plant, such as maize.

The mechanism of discrimination in plants possessing the C4 pathway is such that these plants discriminate less than those with the C3 pathway (Bowman *et al.*, 1989, Ehleringer *et al.*, 1989 and Farquhar (1983)). Models predict that in C4 photosynthesis, isotope discrimination should depend on the ratio of mesophyll intercellular and atmospheric partial pressures of C02 (pi/pa), and leakiness (Farquhar 1983). But contradictory results were also reported, where in, $\Delta^{13}C$ varied significantly in sorghum varieties (Hubick *et al.*, 1989)

In the present study, RILs having similar leaf area were classified on the basis of $\Delta^{13}C$ as low and high $\Delta^{13}C$ types. Physiological traits among these low and high $\Delta^{13}C$ types also varied non significantly (Table 25). There was no relationship between biomass and $\Delta^{13}C$ (Fig 12) suggesting less relevance of $\Delta^{13}C$ in the present context.

On the other hand, traits such as biomass, leaf area, leaf weight, specific leaf area, stem weight, plant height, tiller number, root weight, root length, root to shoot ratio and root volume varied significantly in mapping population (Table 21) and most of the traits revealed continuous variation suggesting their quantitative mode of inheritance (Fig.8)

The total biomass is the sum of the root and shoot dry matter produced by a plant. Further, the biomass produced by a plant is known to be influenced by genetic makeup of the plant, its physiological behavior as well as the external influences like environmental factors. Hence, examining the variations in these traits and assessing the influence on biomass is crucial in crop improvement efforts. Hence mapping population was classified on the basis of biomass as low and high biomass types (Table 22). Except $\Delta^{13}C$ and SLA, other biometric traits varied significantly.

Assessment of contribution of physiological traits other than the canopy cover that determine the variability in biomass is best done when genotypes with similar leaf area but differing in biomass are compared. All the traits varied significantly between low and high biomass types, except $\Delta^{13}C$ (Table 24).

Relationship between biometric traits in the mapping population of finger millet

Being quantitative in nature, the physiological and biometric traits recorded in the mapping population are influenced not only by the environmental factors but also the plant's genetic constitution. Hence, it is important to know the interrelationship among these traits that contribute to the total biomass of the plant when breeding programme targets yield improvement

Many root and shoot traits were positively correlated with biomass (Table 27) indicating their contribution to the total biomass. But inverse relationship observed between biomass and root to shoot ratio (Fig.11) suggests that plant allocates carbon reserve to root at the cost of shoot growth. In a nutshell, it can be inferred that for further investigation in this mapping population it would be better if the selection is made for biomass and root traits.

The complex traits which are under the control of poygenes cannot be easily traced. The advent of molecular marker technology in the 1980s has opened a new era for quantitative genetics studies and with an appealing prospect of its use in breeding program. Over the past 10 years, molecular markers have been extensively used to identify QTLs involved in the expression of traits of agronomic importance (Lee, 1995). This include the genetic dissection of yield components (Kraja and Dudley, 2000; Austin *et al.,* 2000), resistance to disease (Moon *et al.,* 1999; Welz and Geiger, 2000) and, to a lesser extent tolerance to abiotic stresses, especially crop production under water limited environments (Ribaut and Poland, 2000).

Genetics of traits among the RILs of finger millet

The main difference between quantitative and qualitative traits lies in the degree to which they are affected by the environment. Qualitative characters are little or not at all affected by the environment while quantitative characters are considerably affected. The main effect of environment is to mask the difference between different genotypes and to produce a continuous variation in the character. Thus the environmental effects would produce a continuous variation even if the number of genes governing the character were very small or even one. A similar continuous variation will be produced if the number of genes governing the character is increased and affected by the environment. As expected physiological traits showed a continuous variation confirming the metric nature of the trait (Fig 13).

Among these traits LA was the most prominent contributor to biomass. However, at a given leaf area, physiological traits like WUE and other biometric parameters like root weight significantly contributed to the biomass. However it would become essential to assess the heritability of these traits before attempting to exploit these traits in breeding program. An effort was made to assess the genetics of these traits.

The effectiveness of phenotypic selection largely depend on the genetic control of the trait. Therefore to know the extent of genotype and environmental effect on phenotype, genetic parameters like Genotypic coefficient of variation (GCV), Phenotypic coefficient of variation (PCV), Broad sense heritability (H^2) and the Genetic advance (GA) as per cent mean were calculated for various biometric traits. The concept of heritability explains whether differences observed among individuals arose as a result of differences in genetic makeup or due to environmental forces. All biometric traits showed a high heritability except plant height, while the tiller number showed a moderate heritability. Nanak Chand and his associates in 2008 also showed that plant height and tiller number revealed low heritability. Highest heritability was noticed in root volume (95.97%) followed by $\Delta^{13}C$ (94.8%). The GCV and PCV for all the traits were moderate to high except for SCMR, $\Delta^{13}C$ and tiller number for which both GCV(4.18, 7.90 and 3.47 respectively) and PCV were low (4.98, 8.17 and 8.3, respectively). Highest GCV was noticed for root volume and lowest GCV for plant height. PCV for the biometric traits ranged from 4.31 % for MRC to 45.7 % root volume.

Genetic advance gives an idea of possible improvement of new population through selections, when compared to the original population. Genetic advance (GA) as per cent mean was high for all the biometric traits except for SCMR, MRC and plant height. Moderate Genetic advance (GA) as per cent mean was noticed in SLA, $\Delta^{13}C$ and tiller number.

Stability of Physiological traits

For successful molecular breeding, not only accurate phenotyping is important but assessing stability of the physiological traits across the seasons is also crucial. Hence another phenotypic experiment was conducted in the second season. Many traits showed significant positive relationship across the seasons indicating their relevance in genotyping.

Molecular Characterization

In present study, 323 genomic SSRs were developed and further standardized for their annealing temperatures, out of which 175 showed locus specific amplification. The standardized genomic SSRs were screened in parents (IE 2912 and IE 2885) to assess the polymorphism. The polymorphic SSRs were further genotyped in mapping population.

In addition to in-house developed genomic SSRs, genic and outsourced (finger millet and rice) SSR were also standardized in finger millet. But none of them showed polymorphism between parents except few outsourced finger millet SSRs. The low level of polymorphism could be due to the narrow genetic base and most likely went through a bottleneck during domestication which was confirmed through Isozyme, DNA marker analyses including RFLP (Hilu and Johnson 1992; Werth *et al.* 1994; Muza *et al.* 1995; Salimath *et al.*, 1995, Dida *et al.*, 2007)

The present study also involves RAPD analysis which revealed lower level of polymorphism. Initially large number of RAPD markers (315) analyzed for parental polymorphism resulted in only 32 polymorphic markers contributing to 95 polymorphic loci. Further polymorphic RAPD primers were used for genotyping in mapping population.

Linkage analysis in tetraploids

Linkage analysis in autotetraploid species has been an historical challenge in quantitative genetics theory and is a stumbling block that urgently needs to be removed in the rapidly emerging genome research. All tetrploids/polyploids follow tetrasomic/polysomic inheritance. Because of a lack of well established theory for linkage analysis with polysomic inheritance, earlier studies had been based either on the use of simple dominant markers (e.g., AFLPs and RAPDs) that segregate in a simple 1:1 ratio in or use of the corresponding diploid relatives as an approximation to the polyploid case and have not considered important features of tetrasomic inheritance (double reduction and recombination).

Several reasons exist why genetic linkage analysis at a polyploid level is necessary. First, meiotic processes in autopolyploids differ greatly from those in diploids (Bailey,1961). This finding suggests a requirement to take account of the distinct features of gene segregation of autopolysomic inheritance. Secondly, polyploidization and subsequent evolution of polyploid genomes is an extremely dynamic process (Song *et al.*, 1995), implying that it may not be appropriate to approximate a polyploid genome directly with its diploid relative. Thirdly, the diploid relatives of some polyploid species may not exist. Finally, use of more informative genetic markers such as microsatellite markers require modeling the inheritance of multiplex alleles of the polyploids.

Luo and his associates in 2004, proposed successful theoretical model for genetic linkage analysis in autotetraploid species. The model considers both double reduction and recombination, the most essential features of tetrasomic inheritance with linked loci, whereas the statistical method takes appropriate account of the major complexities in analyzing both dominant and codominant molecular marker data during map reconstruction in tetraploid species. These complexities include the problems arising from multiple dosage of allelic inheritance, null allele, allelic segregation distortion, mixed bivalent and quadrivalent pairing in meiosis, and incomplete information of marker-phenotype data. The theoretical analysis established the relationship between the coefficients of double reduction at linked loci, which is essential in the present tetrasomic linkage analysis and in assessing the impact of double reduction on the evolution of tetraploid populations.

Linkage map in finger millet

Genetic linkage maps are necessary tools for genome analysis and breeding applications in crop species. A high-density map can provide information to locate genes, facilitate marker-assisted selection, and clarify the biological basis of complex traits (Grattapaglia and Sederoff, 1994; Sewell *et al.,* 1999). DNA markers facilitate the construction of linkage map, which permit the localization, and evaluation of QTL Although the first genetic linkage map of finger millet was developed by Dida *et al.*, in 2007, but association of markers to different biometric traits has not been done. In present investigation, genetic linkage map was constructed with both co-dominant (SSR) and dominant RAPD marker systems which is a basic prerequisite for association markers to different physiological traits. This is the first report in our study showing association of markers to different physiological traits. Further more, SSRs and other marker systems would permit a better coverage of the genome leading to map saturation.

Association of markers through Single Marker Analysis (SMA)

A significant association between the traits and the markers suggests that a QTL is in the vicinity of the markers. Thoday (1960) proposed the idea of using two markers to bracket regions for detecting QTLs. The detection of QTLs is based on the comparison of the mean values of a given trait computed for a set of individuals grouped by specific marker classes.

A total of 6, 8 and 11 markers were associated with leaf traits like SLA, total leaf area and total leaf weight, respectively. Of all the three traits, SLA had a marker (RAPD marker KITS8c) explaining a highest phenotypic variance of 8.5% at $p<0.0002$. It has been adequately demonstrated that SLA is a convenient alternate for assessing the difference in WUE (Wright *et al.,* 1993; Brown and Byrd, 1996, Rao *et al.,* 1995). Further the relationship between WUE and SLA has been shown to be mediated through the specific leaf nitrogen (Sheshshayee *et al.,* 2006). From this context, the markers associated with SLA can be used in marker assisted breeding for WUE (Xinyou et al., 1998,). The SLA markers were also found to be associated with other leaf traits such as total leaf area (SSR35) and total leaf weight (RAPD marker KITS8a). After proper validation, such markers can be efficiently utilized in MAS for increasing both total leaf area and total leaf weight, which ultimately results in higher photosynthetic capacity for carbon fixation.

Among shoot traits, tiller number associated with maximum of 14 markers with phenotypic variance ranging from 2.45% to 7.76% followed by plant height with 7 markers and stem weight with 2 markers.

Molecular breeding for root traits

A root system that extends the root zone more fully to extract available soil to provide for evapotranspirational demand has the potential to increase yield under drought (Mambani and Lal, 1983). Venuprasad *et al.*, 2002 reported significant positive association of grain yield per plant with high root biomass.

Improving root trait is a potential approach for crop improvement. However, the limitation for this approach being the difficulties associated with the quantification of root traits and large labour requirement for screening these traits such as root pulling force, root penetration ability, etc. (O'Toole and Soemartono, 1981; Zhang *et al.,* 2001; Li *et al.,* 2005). While evolving rice varieties for upland conditions root pulling force or root penetration abilities have little relevance. Therefore it is imperative that the large genetic variation that exists for root length and root biomass be exploited. Nevertheless, it is shown that deep-rooted plants can exhaust soil water resources more quickly than the shallow rooted plants. Deep rootedness hence can be disadvantage in situations where there is little or no rainfall during the crop growth season. In such conditions, the deep-rooted plants will invariably experience end season drought, which might severely affect grain growth and productivity (Condon and Richards, 1993). From this context, it also becomes essential to simultaneously improve the efficiency with which the plants use water.

In the present study, root traits were found to be associated with more number of markers compared to both leaf and shoot traits (10, 13 and 11 markers with root length, root volume and root weight respectively). After validation, the markers explaining higher variance for these traits can be targeted for exploitation for crop improvement

Future Line of work:

Development of SSR markers-Although good number of genomic SSR has been developed in fingermillet, still there is good scope to increase the number.

Map saturation- In the present study, the skeletal genetic linkage map of finger millet has been developed. This map needs to be saturated with more number of molecular markers such as SSRs, AFLP etc.

QTL mapping –In the present study, markers have been associated with different physiological traits through Single marker analysis, furthermore there is a need to identify relevant QTL for different traits.

Summary

VI. SUMMARY

Several diverse and inter related mechanisms bring in increased level of adaptation to moisture stress. One of the pre requisite is to identify the major effect QTL to pyramid by marker assisted selection. One such important trait related to superior adaptation to drought is roots. A dynamic root system is fine-tuned to soil moisture status and is known to regulate the amount of water available to the plant depending on its distribution in the soil. Despite ample genetic variation for many root related parameters, genetic improvement of root characteristics in fingermillet using conventional selection has been difficult and progress towards improvement of yield in rain fed has been hampered. A significant proportion of the phenotypic variability of several of these putative drought resistance traits is explained by the segregation of relatively few genetic loci, thus leading to the possibility of indirect selection of these complex traits by means of marker-assisted selection (MAS) strategy. Considering these limitations to efficient selection, molecular marker technology is a powerful tool for selecting such traits. One of the limitation to achieve this target is lack of robust locus specific marker systems. In view of this, one of the objectives of the present investigation was to develop SSR markers (genomic and genic). The other objectives are phenotyping for drought traits and molecular characterization of recombinant inbred lines of Finger millet by different markers. The results of these experiments are summarized in this chapter.

6.1 Development of microsatellite markers

Highly informative molecular markers, such as simple sequence repeats (SSRs), can greatly accelerate breeding programs**.**

6.1.1 Finger millet genomic SSRs

In the present study, precloning enrichment strategy was followed to fish out microsatellite markers from the finger millet genome, where in the whole pool of digested genomic DNA was subjected to repeat oligomer hybridization, thereby leading to selective hybridization.

- From the enriched library, 2200 colonies were screened using universal M13 primers. About 1021 recombinants with fragments size of 500-1000bp were sequenced and annotated. Of these 645 sequences contained the microsatellite repeats, which varied in repeat type and size.
- The library consisted more of di-nucleotide repeats (67%) followed by mono (11%), tri (14%) tetra (4%) and penta (4%) nucleotide repeats. Among di-nucleotide repeats, the CT-based repeat motifs were the most abundant (30%) followed by AG repeats (21%). Among tri-nucleotide repeats TGA and TCA were found to be abundant Tetra and penta-nucleotide repeats were relatively less compared to mono, di and tri-nucleotide repeats in the enrichment library.
- A total of 323 primer pairs were designed in the flanking regions of microsatellite repeats, of which 156 primers showed locus specific amplification.

6.1.2 Development of genic SSRs from stress specific finger millet EST

To develop genic SSRs, dehydration stress specific EST library constructed by subtractive hybridization at the Department of Crop Physiology, UAS Bangalore was annotated for the presence of microsatellite regions by the web based programme-Tandem Repeat Finder. These Simple sequence repeats developed from the existing finger millet EST sequences could act as genic SSRs a powerful marker system as these are present in coding region of the genome. From the annotation of 796 dehydration stress specific ESTs, 149 genic SSR primers were developed, of which 87 primers showed locus specific amplification.

6.1.3 Validation using finger millet SSRs from public domain/ database

All the 31 fingermillet SSR primers from the public domain locus specific amplification.

6.1.4 Validation using rice SSRs from public domain/ database

Cross species amplification was studied with 228 rice SSR primers. Of these, 69 showed locus specific amplification.

6.1.5 Locus specific SSR markers generated in this study

Totally 323 genomic and 149 genic SSRs were developed. Among them, 156 genomic and 87 genic SSRs showed locus specificity, and these markers are submitted to NCBI database, which could be used for genotyping studies in finger millet. In addition, outsourced 31 finger millet SSR and 69 rice SSR markers also available for genotyping and synteny studies in finger millet

6.2 Identification of molecular markers for root traits, WUE and other physiological parameters in finger millet

To identify relevant QTL of interest molecular markers and suitable mapping population are pre requisites. Two parents, IE 2912 and IE 2885 contrasting for drought and neck blast resistance, were crossed and 150 RILs (recombinant inbred lines) developed and were used for both phenotyping and genotyping studies.

6.2.1 Phenotyping in recombinant inbred lines of finger millet

6.2.1.1 Experiment 1

Recombinant inbred lines along with the parental lines were phenotyped by raising the plants in specialized root structure under the natural field conditions. The diverse growth and physiological parameters were recorded, especially the comprehensive root characteristics at flowering time, besides growth parameters like plant height, tiller number, biomass were measured. In addition, $\Delta^{13}C$ values were recorded to assess the variability in WUE. RILs were also phenotyped for SCMR and MRC.

- Parent IE 2912 showed better performance in many traits than IE 2885 except SCMR, SLA, MRC and $\Delta^{13}C$.
- Different physiological traits such as biomass, total leaf area, plant height, tiller number, $\Delta^{13}C$, SCMR, MRC, root traits such as root length, root volume, root weight and all the traits varied significantly among the RILs except $\Delta^{13}C$, SCMR and MRC. Further the traits showed normal distribution around the mean and continuous variation indicating that the traits are quantitative in nature.

Being quantitative in nature, the physiological and biometric traits recorded in the mapping population are influenced not only by the environmental factors but also the plant's genetic constitution. Hence, it is important to know the interrelationship among these traits that contribute to the total biomass of the plant when breeding programme targets yield improvement.

- A strong positive correlation was observed between the leaf area and the BM suggesting that the photosynthetic surface area contributes significantly to the BM production.
- A significant positive correlation between root and shoot dry weight indicated that the root biomass significantly contributes to the shoot growth.
- However an inverse relationship was observed between root to shoot ratio and the shoot dry weight suggesting that in high biomass types the relative allocation to roots is less.
- Though a strong positive association was noticed between leaf area and biomass, $\Delta^{13}C$ did not show any relationship with BM indicating that the higher in biomass in these accessions is mainly through increased transpiration associated with leaf area and root traits.
- To Assess of contribution of physiological traits other than the canopy cover that determine the variability in biomass is best done when genotypes with similar leaf area but differing in BM are compared. The two groups of genotypes despite little differences in leaf area, differed significantly for BM suggesting that net assimilation rate contributing for the variation.
- In high BM types, traits such as root weight, root volume, shoot weight and leaf weight were found significantly higher than low BM types.
- There was no significant difference in $\Delta^{13}C$ between low and high BM types signifying that difference in biomass is mainly due to total transpiration but not WUE.

6.2.1.2 Experiment 2

The second Phenotyping experiment has been done in similar way as that of first phenotyping experiment to reconfirm the variability in different physiological traits in the mapping population and also to assess the stability of traits across the season. The observed continuous variability in several physiological traits suggests the possibility of identifying markers associated with the traits. Significant positive correlation was seen for number of growth parameters like total biomass, root characteristics and also tillering ability between two seasons. The results showed inspite of G X E interaction, there is genotypic consistency in several growth parameters across the seasons.

6.3 Molecular characterization

6.3.1 Parental polymorphism

Parental polymorphism studies were conducted with finger millet genomic and genic SSRs, outsourced rice SSR and RAPD markers.

In house developed genomic SSR primers were screened between the two parents (IE 2912 & IE 2885) to study polymorphism.

- Out of 156 locus specific markers, ten SSR primers showed polymorphism between the parents.
- None of the 87 EST-SSRs were polymorphic between the parents.
- Five out of 31 outsourced Finger millet SSR primers revealed parental polymorphism.
- None of the outsourced rice SSR primers revealed polymorphism between the parents.
- About 315 RAPD primers were screened between the two parents, out of which 32 primers revealed polymorphism contributing 95 polymorphic loci.

6.3.2 Genotyping the recombinant inbred lines

- The polymorphic SSRs and RAPD markers were further screened in the RILs. Totally 15 polymorphic SSRs and 95 RAPD marker loci were used to construct linkage map.

6.4 Linkage map construction

The polymorphic markers were run on the individuals of the RIL population in order to generate the marker data for linkage map construction. A skeletal linkage map was constructed with 95 RAPD and 15 SSR marker loci using 150 RILs, using the software MAPMAKER/EXP 3.0. The linkage groups were created with a LOD score of 3.0 and with a maximum distance threshold of 50cM which formed 14 linkage groups. A minimum of 4 markers mapped on the linkage group 14 and a maximum of 9 markers covered the linkage groups 3 and 4. In total, 102 markers covering 6519.4 cM on 14 linkage groups were mapped.

6.3.2 Association of Markers/QTLs for physiological traits

A total of 6, 8 and 11 markers were associated with leaf traits like SLA, total leaf area and total leaf weight respectively. Among the shoot traits, tiller number associated with maximum of 14 markers followed by plant height with 7 markers and stem weight 2 markers. Root traits were found to be associated with more number of markers compared to both leaf and shoot traits. Root length, root volume and root weight were found to be associated with 10, 13 and 11 markers respectively.

Outcome of the study

Identifying QTLs for diverse drought adaptive traits is a relevant approach and forms the basis to introgress them by marker assisted selection. However, the primary requisite for mapping the QTL is developing a linkage map. In this regard, lack of locus specific SSR markers is a major constraint. From this context, one of the contributions of this study is generation of SSR marker systems, both genomic and genic which can be used by the diverse groups for molecular characterization of finger millet. Overall 156 number of genomic SSRs, 87 genic SSRs and 69 rice SSRs are now available for genotyping studies,

The study also showed that significant genetic variation exists among 150 recombinant inbred lines for diverse physiological traits especially water mining and water conservation traits. The present study also clearly demonstrates that the existing polymorphism for many of the locus specific markers is relatively poor signifying the need to develop more polymorphic markers like SNPs. Further utility of these results depends on further genotyping of the RILs with diverse marker systems.

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