University of Alberta

Effects of drought on *Eleusine coracana* (L.) Gaertn. (finger millet) and identification of microsatellite markers

by

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Abstract

Finger millet (*Eleusine coracana* (L.) Gaertn.) is a crop with high nutritional profile cultivated mainly in developing countries. It is an allotetraploid (2n= 4x= 36 chromosomes), that belongs to the Poaceae family and has a genome size of 2509 Mbp. Drought is one amongst other abiotic stresses limiting a plant's growth and productivity. In order to better understand the physiological responses of finger millet to drought, 12 parameters were studied by subjecting plants to water withholding at 26 DAS and imparting water stress for 6 days. Analyzing control plants in parallel indicated that IE 7079 was the most sensitive accession and IE 5091 was the most tolerant accession to water withholding. 83 accessions of finger millet obtained from ICRISAT was genotyped using 31 polymorphic simple sequence repeats (SSRs) identified from transcript assemblies. A total of 152 alleles were generated by these 31 SSRs, with a mean of 4.9 alleles per locus and mean polymorphism information content (PIC) of 0.49 Cluster analysis of these accessions showed considerable genetic variation in the varieties from different geographical origins. Identified polymorphic SSRs can be used to further expand the linkage map of finger millet.

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List of Abbreviations

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
AISMP	All India Small Millets Improvement Programme
AP-PCR	Arbitary primed PCR
APX	Ascorbate peroxidase
BGI	Beijing Genomics Institute
cDNA	Complimentary DNA
Chl_a	Chlorophyll a
Chl_b	Chlorophyll b
C_i	Internal CO_2 concentrations
CTAB	Cetyl trimethylammonium bromide
C_{x+c}	Total carotenoid
$CytP_{450}$	Cytochrome Plasmid
DAF	Amplification fingerprinting

DAS	Days after seeding
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DW	Dry weight
E	Transpiration rate
EC	Electrical conductivity
EL	Electrolyte leakage
EMBL	European Molecular Biology Laboratory
EST	Expressed sequence tag
FAO	Food and agricultural organization
FC	Field capacity
FW	Fresh weight
GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
g_s	Stomatal conductance
HCl	Hydrochloric acid

ICRISAT	International Crops Research Institute for Semi-Arid Tropics
ISSR	Inter simple sequence repeat
ITS	Internal transcribed spacers
Kbp	Kilo base pair
KCl	Potassium chloride
LEA	Late embryogenesis abundant
MAS	Marker assisted selection
Mbp	Million base pair
MSSRF	M.S. Swaminathan Research Foundation
NCBI	National Center for Biotechnology Information
NR	Nitrate reductase
NRC	National Research Council
PCR	Polymerase chain reaction
pDr	Progressive drought
PIC	Polymorphism information content
P_n	Net photosynthetic rate

PPF	Photosynthetic photon flux
Ppm	Parts per million
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RT-PCR	Real time PCR
RuBP	Ribulose-1, 5-bisphosphate
RWC	Relative water content
SD	Standard deviation
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SRA	Sequence Read Archive
SSLP	Simple sequence length polymorphism
SSR	Simple sequence repeat
SSRP	Simple sequence repeats polymorphism

STMS	Sequence tagged microsatellite sites
STR	Short tandem repeat
Taq	*Thermus aquaticus*
TC	Total chlorophyll
TW	Turgid weight
USDA	U.S. Department of Agriculture
UVB	Ultra violet B
WUE	Water use efficiency

Chapter 1 – Literature Review

1 Introduction

1.1 Finger millet

Finger millet, or ragi, (*Eleusine coracana* (L.) Gaertn.), is a food crop mainly cultivated in Africa and Southern Asia. The genus *Eleusine* is a member of tribe Eragrosteae, subfamily Chloridoideae, family Poaceae, and contains about 12 species, of which *E.coracana* is the only one of economic importance (Bisht and Mukai 2002). Finger millet is phylogenetically closer to tef (*Eragrostis tef* L.) than to other millets (e.g. pearl millet (*Pennisetum typhoides* S. & H.), fox-tail millet (*Setaria italica* Beauv.), proso millet (*Panicum miliaceum* L.), kodo millet (*Paspalum scrobiculatum* L.), and little millet (*Panicum miliare* Lam.)), which are all part of the Panicoideae subfamily.

Finger millet (Fig. 1.1a) is an annual crop that grows 96-135 cm in height and takes about three to six months to mature. It is C_4 crop that has an erect, compressed and glabrous stem housing the leaf blades which are linear, folded and striated, often having ciliated margins (Dida and Devos 2006). The inflorescence (Fig. 1.1b) resembles fingers on a hand, hence its common name "finger millet". Each spike contains about 70 spikelets arranged alternately on the rachis. Their seeds vary both in shape (circular and ovular) and color (red, black, white, and orange). *E. coracana* has well developed spikes that are straight, slender and up to 11 cm in length. Finger millet breeding is mainly confined to southern India, eastern and southern Africa (Dida and Devos 2006). Average

yields between 1000 and 5000 kg ha^{-1} under various irrigation conditions have been reported (Fig. 1.2).

(a)

(b)

Figure 1.1: (a) Finger millet growing in the fields of Koraput District, Orissa, India. Courtesy: Dr. Michael Deyholos (b) Inflorescence in finger millet

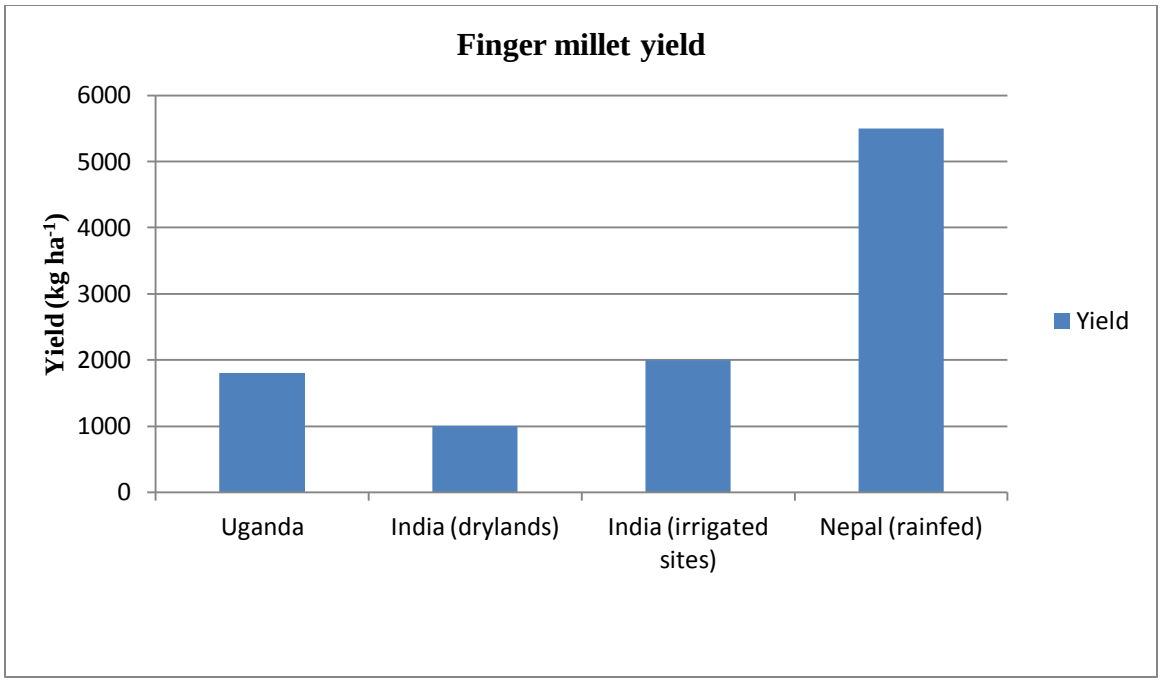

Figure 1.2: Yield of finger millet. Yields recorded in different countries under various irrigation conditions for the year 1996 (National Research Council, India 1996). Finger millet grows best in semi-arid environments with minimal rainfall and an annual temperature of 11-29°C. Well-drained soils are favorable, but silt loams are the most desirable. Soils with a pH of 5.0-8.2, best supports its growth.

1.1.1 Genome donors

Finger millet is an allopolyploid believed to have arisen in the highlands of eastern Africa through spontaneous hybridization of *Eleusine indica* (now a major weed) and another species of the same genus (Hilu, De Wet et al. 1979; Hilu and Johnson 1992; Liu, Triplett et al. 2011). Figure 1.3 shows the evolutionary pathway of the species *E. coracana*. It has been established that the A genome donor is the diploid, *E. indica* (Dida and Devos 2006). However, the identity of the B genome donor is still a subject of some debate. Based on

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

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

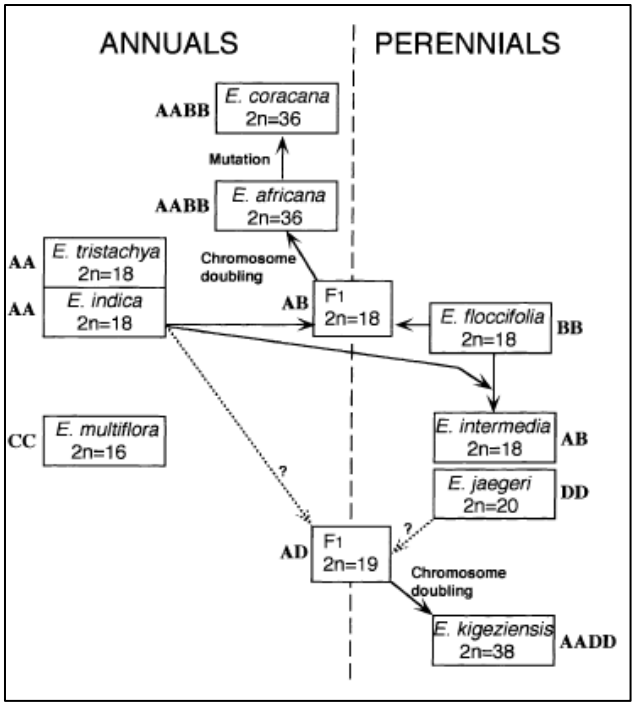

Figure 1.3: A proposed pathway for the evolution of *Eleusine coracana*. The allotetraploid, *Eleusine coracana* (L.) Gaertn is believed to have originated through crosses between diploid and polyploid species of the genus *Eleusine* (Bisht and Mukai 2002).

1.1.2 Genome size

Finger millet is a tetraploid with a genome constitution of AABB, and base chromosome number of 9 (*2n = 4x = 36)*. In 1991, the amount of nuclear DNA for 30 collections belonging to 10 species of *Eleusine* were determined (Hiremath and Salimath 1991). Congeners of *E. coracana*, showed a 1C DNA content range from 2.55 pg in *E. africana* to 3.08 pg in *E. floccifolia* (Bennett 2012). Later in 1995, the genome size of the tetraploid crop was estimated to be 2.90 pg by Feulgen microdensitometry (Mysore and Baird 1997). Most recently, the finger millet genome size has been measured as 1.8 pg/1C nucleus by flow

cytometry (Dida, Ramakrishnan et al. 2007), corresponding to approximately 2509 Mbp (Bennett and Leitch 1995). This is larger than the sequenced 430 Mbp rice (*Oryza sativa*) genome (Pennisi 2007) but smaller than the 17000 Mbp wheat (*Triticum aestivum*) genome (Brenchley, Spannagl et al. 2012).

1.1.3 Origin and distribution

Finger millet is believed to have originated in Africa, 5000 years ago. It then made its way through Western Uganda and the Ethiopian highlands of Eastern Africa (Dida and Devos 2006). Soon after, the crop was domesticated to India whereupon the subcontinent became its secondary centre of diversity. It is also cultivated in Burma, southern parts of Tibet, Nepal, Malaysia, Sumatra, Sri Lanka, Philippines, Japan, China, Java, Iran, Afghanistan and in the Arabian peninsula (Bisht and Mukai 2002). Most of the species are confined to Africa, however, *E. coracana* has made its way through Africa and the Indian subcontinent. Table 1.1 outlines the distribution of various *Eleusine* species.

Table 1.1: Distribution of *Eleusine* species along with their growth habitat (Bisht and Mukai 2002).

Species	**Growth habitat**	**Geographical distribution**
E. indica	Annual	Japan, India, North America, Tanzania, Belgium, Hungary
E. tristachya	Annual	South America
E. multiflora	Annual	Kenya, Tanzania

E. floccifolia	Perennial	Ethiopia, Somalia, Kenya, Yemen
E. intermedia	Perennial	Ethiopia
E. jaegeri	Perennial	Tanzania
E. kigeziensis	Perennial	Burundi
E. africana	Annual	Malawi, Kenya, Rhodesia, Tanzania
E. coracana	Annual	India, Nepal, Uganda, Kenya, Ethipoia

1.1.4 Germplasm collections

As finger millet gained importance as a food crop, several national research programs in different countries assembled germplasm collections. While each country was storing its native finger millet lines, it was the International Crops Research Institute for Semi-Arid Tropics (ICRISAT) that began assembling germplasm in 1976. Their collection now holds 5,949 accessions from 23 different countries (Upadhyaya 2007). The Agricultural Research Station of the USDA in Georgia maintains 776 accessions belonging to 11 countries. The National Bureau of Plant Genetic Resources, based at New Delhi, India maintains 10,507 accessions. The All India Small Millets Improvement Programme (AISMP) at Bangalore, India, collects and preserves millet germplasm to meet national researcher's needs. The following countries hold collections of millet germplasm as well: Nepal (877 accessions), Sri Lanka (393), Bhutan (84), Kenya (1902), Zimbabwe (1158), Uganda (1155), Zambia (497), Tanzania (293), Malawi (145), Eritrea (120), Burundi (113), Ethiopia (71), Nigeria (20), South Africa (17), China (300), Russian Federation (110) and Vietnam (52) (ICRISAT, 2012).

1.1.5 Uses of finger millet

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

Finger millet has a better nutrient profile (Table 1.2) than rice, corn or sorghum in terms of protein, fat, minerals and essential amino acids. The main storage protein (elusinin), contains good amounts of tryptophan, cysteine, methionine, and total aromatic amino acids, which are all crucial to human health and growth, but are deficient in most major crops (NRC, 1996). Because of such attributes, finger millet has been promoted as part of a solution to malnutrition, especially in parts of India (Singh and Raghuvanshi 2012). Essential amino acids namely, tryptophan, threonine, lysine, methionine, valine and isoleucine were found to be present in great proportions in finger millet (FAO, 1991). The crop is

also reported to have hypoglycemic, hypocholestrolemic and anti-ulcerative properties (Panwar, Saini et al. 2010).

Table 1.2: Nutritional profile of finger millet (Singh and Raghuvanshi 2012).

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

1.1.6 Agronomic importance of finger millet

Apart from its strong nutritional value, finger millet is also popular with farmers because it is adaptable, resilient, and yields well on marginal land without irrigation (Kumar, Gangwar et al. 2002). With its excellent storage qualities and tolerance to alkali, high pH, fungus, insects, salt and soil moisture, this crop is comparatively easy to maintain without expensive inputs. It is cultivated over a wide range of soils ranging from rich loams to poor shallow upland soils (Rao and Krishnamoorthy 1981).

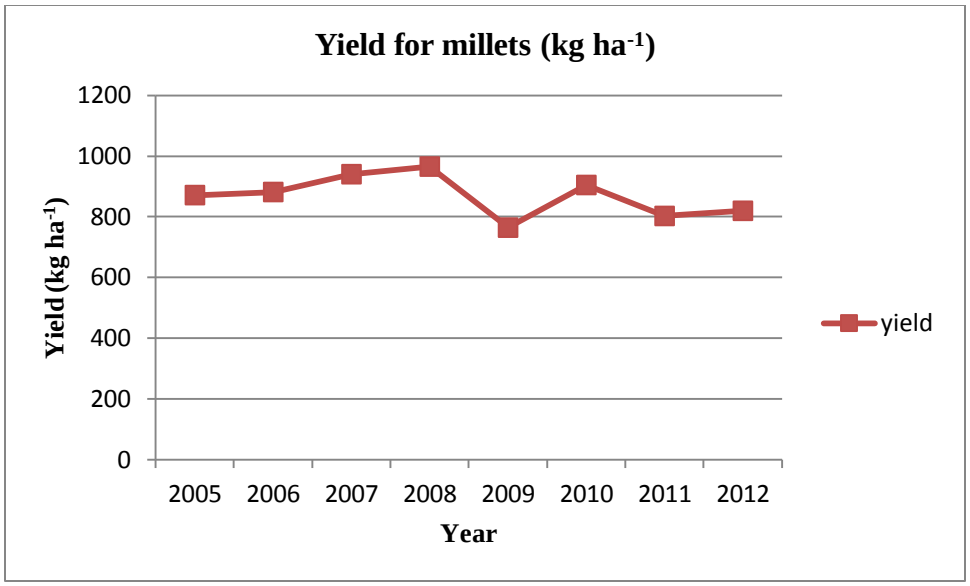

Figure 1.4: Global production statistics of various millets during the year 2005-2012. The average global area harvested for millet in 2012 is about 31 million hectare, yielding ~819 kg ha^{-1} (FAOSTAT 2013)

Production statistics (Fig. 1.4) for finger millet are often lumped together with other millets (sometimes sorghum), thus obtaining reliable estimates is difficult. In 2000, the average global area under millet cultivation was about 37 million hectares which has declined to ~31 million hectares in 2012 (FAOSTAT, 2013). In Zambia, this crop is cultivated in high rainfall regions, yielding about 1000-2000 kg ha^{-1}. In Zimbabwe, finger millet has been cultivated from archaeological times and is grown in regions where rainfall ranges from 450-750 mm yr^{-1} with frequent droughts. Kenya yields about 3800-4000 kg ha^{-1} annually (Oduori 2005). India reported a yield of 5000-6000 kg ha^{-1} under ideal irrigation conditions (National Research Council, 1996). Yields between 400-2000 kg ha^{-1} have also been reported with unimproved varieties (Dida, Ramakrishnan et al.

2007). While efforts were taken to identify blast resistant pearl millet, using comparative genomics, breeding objectives and classical mapping efforts (Devos, Hanna et al. 2006), finger millet was largely overlooked.

In 2011, the University of Alberta (UoA) and M.S. Swaminathan Research Foundation (MSSRF), a non-profit research organization in India, defined an objective to enhance food and nutritional security for the rural poor in agro-biodiversity hotspots with attention to under-utilized crops. The three hotspots in India are: Koraput (Orissa), Wayanad (Kerala) and Kolli Hills (Tamil Nadu). The aforementioned attributes of finger millet make it an ideal crop for our studies. Our objective is to develop molecular tools to aid in the classification of over 2459 finger millet germplasm collection in ICRISAT. This will help plant breeders in identifying sources of genetic diversity.

1.2 Stress physiology of plants

A stress factor (stressor) is a stimulus of biotic or abiotic origin ranging from herbivory and pathogenesis to deficiencies of water and nutrients. Responses to the stressor vary between species, and also vary within species depending on factors like age, degree of adaptation and seasonal activities (Larcher 2003).

Effects of environment/stress on plants may be divided into two categories: enforced damage effects controlled by environment, and adaptive responses controlled by plant. Occurrence of damage to a plant indicates lack of resistance on the part of the plant. Thus, a plant's response to this damage can be considered as adaptations in molecular terms where changes in molecules and

their molecular structure take place or adaptations in morphological or behavioral terms where adjustments to temporal and spatial changes in the environment take place (Fitter and Hay 2001).

1.2.1 Drought

Drought is an extended period of time during which a region is under limited water supply. Limited water availability to plant cells can be due to physical and climatic characteristics of the environment, soil-precipitation relationship, soil-plant relationship, the atmosphere-plant relationship, excessive demand by the plant, or any combination of these. Drought is a major environmental constraint on the productivity of many crops, and affects both crop quality and the quantity of the yield (Bradford and Hsiao 1982).

Studies have been conducted in various species to show the responses of plants to drying environments (Bray 1993; Shinozaki and Yamaguchi-Shinozaki 1997; Rao, Raghavendra et al. 2006; Bhatt, Negi et al. 2011). Most of these studies indicated that in order to protect the plant against water loss due to transpiration, they close their stomata. This closure can be abscisic acid (ABA)-dependent or independent. Loss of leaf water potential will, in turn, affect the turgor pressure of leaf tissues, further affecting the photosynthetic apparatus (Rao, Raghavendra et al. 2006). Since there are a number of responses of a plant to drought stress, a review was conducted in an attempt to correlate biochemical events with the physiological parameters (Flexas and Medrano 2002). It was noticed that a decrease in some biochemical activities corresponded fairly well to a decrease in stomatal conductance, but not to a decrease in relative water content

(RWC). It was also found that the first biochemical step impaired during drought is ATP synthesis and mild effects on ribulose-1, 5-bisphosphate (RuBP) regeneration.

In addition to morphological and physiological responses of plants to water stress, a complex set of responses is also elicited at cellular and molecular levels. In the 1990's efforts were first taken to understand the molecular mechanism behind these responses. Changes in gene expression are fundamental to the responses that occur during water deficit. Studies have identified multiple changes in gene expression by 2D-PAGE and screening of cDNA libraries, in order to study the function of drought-induced gene products and their pathways which lead to gene induction (Bray 1993).

Another review outlined the molecular mechanisms to drought tolerance in higher plants (Farooq, Wahid et al. 2009). During drought, the metabolic and morphological changes experienced by plants are accompanied by changes in gene expression. Microarray studies in 7000 genes in *Arabidopsis* under drought stress revealed 277 up-regulated genes and 79 down-regulated genes (Seki, Narusaka et al. 2002). Gene expression can be triggered either directly or as a result of a secondary stress response or injury response. A number of stress related genes have been identified, however their complex phenomena of whole molecular response to stress is still unclear (Cattivelli, Rizza et al. 2008). Transcriptome analysis using microarrays and RNA-seq are now being employed to reveal the multifaceted dynamics of transcriptome (Deyholos 2010).

1.2.2 Genes related to drought stress

One of the practical challenges in studying molecular responses to water stress is the environment. Studies on many different plants have shown that plants respond differently in growth chambers, green houses and the field. Sometimes, stress applied in laboratories may not accurately represent the same intensity as the field. Laboratory stresses are rapid and severe, whereas in the field, a plant experiences stress over an extended period of time (Van Volkenburgh and Davies 1977; Radin 1992).

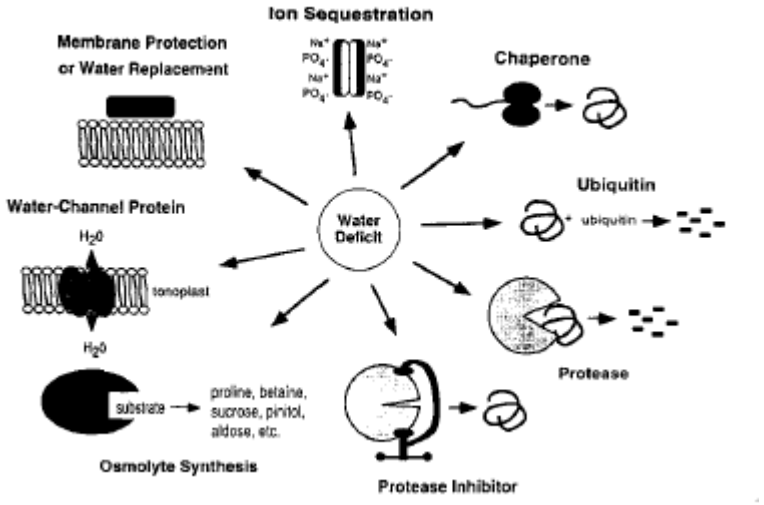

Figure 1.5: Genes induced during stress. Genes that function during changes in metabolism, regulation, signaling, and recognition of stress are expected to be induced under stress. Many water-deficit-induced genes encode products predicted to protect cellular function (Bray 1993)

At the molecular level, responses to water deficit are controlled by an array of genes with many different functions. Figure 1.5 outlines the predicted

functions of some water-deficit-induced gene products that may act to maintain cellular function during periods of water loss. Many genes that respond to drought with a change in transcript abundance have been identified. They include water channel proteins; enzymes required for biosynthesis of various osmoprotectants; protective proteins such as late embryogenesis abundant (LEA) proteins; detoxification proteins; and transcription factors which are involved in further regulation of signal transduction and gene expression (Shinozaki and YamaguchiShinozaki 1996).

A large number of genes are up-regulated in response to drought stress (Rao, Raghavendra et al. 2006). These up-regulated genes are categorized into two groups. One includes genes encoding proteins whose catalytic activities are responsible for protecting the cells and organs against stress, while the other includes genes encoding proteins necessary for signal transduction and regulation of gene expression. Further, the drought-responsive genes can be divided into two groups: Abscisic acid (ABA)-dependent and ABA-independent genes (Yamaguchi-Shinozaki and Shinozaki 2006). ABA is a major signal, which is synthesized from carotenoids by ABA-synthesizing enzymes induced in the root tip cells or parenchyma cells of vascular bundles by stress. Expression of gene encoding abscisic aldehyde oxidase along with four other enzymes have been revealed in guard cells of dehydrated Arabidopsis leaves (Koiwai, Nakaminami et al. 2004). These induced genes are thought to function not only in protecting cells from water deficit by production of metabolic proteins but also in the regulation

of genes for signal transduction in the water stress response (Shinozaki and Yamaguchi-Shinozaki 1997).

Extensive studies have been conducted to understand the cellular and molecular responses of both *Arabidopsis* and rice to water stress (Harb, Krishnan et al. 2010; Rang, Jagadish et al. 2011). However, crop species that are naturally well-adapted to drought, such as finger millet, have not been explored much (Ramegowda, Senthil-Kumar et al. 2012).

1.2.3 Effect drought on growth

Disruption in the water status of the plant has consequences on its growth pattern. Since cell expansion is dependent on turgor potential, developing cells expand less and cell size is smaller under water stress. However, the consequences on the growth pattern are dependent on the timing of water limitation with relation to plant phenology (Nilsen and Orcutt 1996). If water limitation occurs at the beginning of the growth cycle, leaf area will be reduced and carbon gain throughout the growing season will be reduced because of smaller leaves. If the same occurs during inflorescence development, the number of flowers is reduced and possibly all reproductive effort may be aborted. The transition phase between the G_1 and S phases of the cell cycle is interrupted by water stress, thus reducing the rate of cell division. The plant's growth pattern is affected by reductions in carbon accumulation, cell number and tissue expansion. Decreased root hydraulic conductivity and a decreased rate of water transport limits leaf expansion (Tardieu, Granier et al. 2011). The imbalance between cell

division and tissue expansion resulted in cell size decrement in Arabidopsis (Hummel, Pantin et al. 2010). Unavailability of water finally causes an irreversible wilting and the plants eventually die (Šebánek 1992).

1.2.4 Effect of drought on cell structure

Cell walls have unique roles in allowing growth to occur under water deficit stress. Turgor pressure is directly proportional to water loss. Plants respond by either relaxing or tightening their cell wall in response to change in turgor pressure. Non-essential tissues are tightened, whereas, important growing points, such as apices, continue to grow at low turgor pressures (Wu and Cosgrove 2000). Studies on maize roots subjected to water stress have shown that apical regions of the cell wall continue to grow at low water potentials, whereas, the elongation region cell walls cease further growth (Fan, Linker et al. 2006). Drought also affects enzymatic processes, pH, expansins, concentration of ABA, solute accumulation, and variation of sugars in cell walls, which in turn affect cell wall loosening or tightening (Moore, Vicré-Gibouin et al. 2008).

Impact of water limitation has also been observed in the ultra structure of cells. Water deficit might disrupt the structure of micro-bodies, releasing hydrolyzing enzymes into the cytoplasm. The presence of these enzymes (lipases and proteases) further disrupt the normal structure of all cytosolic membranes (Nilsen and Orcutt 1996). Water deficit also has effects on the structure of chloroplast and mitochondria. Thus water stress affects germination and seedling growth (Farooq, Wahid et al. 2009).

1.2.5 Effect of drought on photosynthesis

The initial impact of drought on photosynthesis is usually stomatal closure. Stomata may close due to a root signal, probably abscisic acid (ABA), or low turgor pressure in guard cells or in response to an increasing vapor pressure gradient between leaf and air. It is believed that during initial phases of water deficit, stomatal closure and non-stomatal inhibition of photosynthesis occur concurrently (Nilsen and Orcutt 1996). Stomatal closure causes depletion of intracellular CO_2, which in turn stimulates photorespiration. However high light intensity causes photo-inhibition to occur, resulting in the buildup of free radicals in the chloroplast. Non-stomatal inhibition of photosynthesis can be attributed to photo-inhibition and activity of rubisco, depending on the taxa. Photosynthesis is more resilient to water stress than is cell expansion (Hummel, Pantin et al. 2010).

1.2.6 Effect of drought on carbohydrate metabolism

With progression of drought, the ratio of photosynthesis to respiration decreases, with a potential increase in both photo-respiration and dark respiration, owing to plant starvation. It is believed that sugar concentration in some plant tissues may increase with water deficit as starch stored in chloroplasts is mobilized (Nilsen and Orcutt 1996). Resource allocation patterns vary during water deficits. In many species, root growth dominates leaf tissues, thus a decrease in root/shoot ratio.

Carbon fluxes in plants are altered during water stress. Arabidopsis when subjected to soil water deficit showed that biomass growth and leaf expansion decreased, which led to a more positive C balance, whereas root growth increased and photosynthesis was maintained (Hummel, Pantin et al. 2010). Osmotic

adjustments were brought about by accumulation of C metabolites and K^+ at different developmental stages, while nitrate concentration remained constant.

1.2.7 Effect of drought on nitrogen metabolism

Nitrate and ammonia accumulation decrease during water deficit. Flow of nitrogen from roots to leaves slows down under stress conditions, thus leading to accumulation of nitrogen ions in the roots. This in turn inhibits the uptake of nitrogen from soil (Nilsen and Orcutt 1996). Enzymes associated with nitrogen metabolism, namely nitrate reductase (NR), glutamine synthetase (GS), and glutamate dehydrogenase (GDH), are also involved in photosynthesis and carbohydrate metabolism. Reports have shown that these key enzymes have a role to play in the photosynthetic acclimation of plants to drought stress, particularly in late growth stages (Xu and Zhou 2006).

1.3 Molecular markers

Molecular markers are based on polymorphisms detected at the level of macromolecules. DNA-based markers are sequence variants associated with a specific location within a genome. These sequence polymorphisms can take many forms; for instance, insertions, deletions, or substitutions (Henry 2001).

DNA markers are useful for crop improvement, gene introgression through backcrossing, germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis, etc. (Jain and Brar 2010).

A number of marker systems (restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs) and microsatellites) have become available since the 1980s. However, the choice of marker system is largely dictated by the intended application, convenience and the cost involved. Each of the major types of DNA markers is described in the following sections.

1.3.1 Restriction fragment length polymorphism

RFLP analysis depends on the digestion of a DNA sample with a specific restriction endonuclease, which can allow detection of DNA polymorphisms based on the presence of fragments of different lengths. These markers are highly locus-specific and co-dominant, i.e. both alleles in heterozygous sample will be detected. On separating the digested DNA by gel electrophoresis, RFLP probes are developed by labeling DNA sequences that hybridize with one or more fragments. These probes are frequently used in genome mapping and variant analysis.

RFLP markers are generally phenotypically neutral (Tanksley, Young et al. 1989). Like other DNA marker systems, RFLPs are free from epistatic effects and thus can be unequivocally scored in the same segregating population. However, there are considerable disadvantages with this technology. It is time consuming, labor intensive and not a rapid method for evaluation of large segregating populations. Moreover, RFLP analysis requires large quantities of genomic DNA, generally 5-10 μg per digest (Jain and Brar 2010).

One of the first uses of RFLPs in mapping was the generation of a linkage map for *Arabidopsis thaliana* (Nam, Giraudat et al. 1989). RFLP maps have been developed for many crops as well (Tanksley, Young et al. 1989). In 1994, the first genetic map of pearl millet was published consisting of seven linkage groups (Devos, Hanna et al. 2006). For finger millet, as many as 126 RFLP probes were reportedly placed on a linkage map, although this map was never published outside of a thesis (Dida 1998).

1.3.2 Random amplified polymorphic DNA

RAPDs were the first arbitrarily primed PCR markers to be developed. Arbitrary Primed PCR (AP-PCR) and Amplification Fingerprinting (DAF) markers were the other PCR based molecular markers to have developed during the same time. The basic principle behind RAPD is the presence or absence of same arbitrary or random sequence in inverse orientation within an amplifiable distance, so that the same sequence works as forward and reverse primers at multiple loci. The primer sequence for RAPD markers are ~10 nucleotides long (Jain and Brar 2010).

Since RAPDs have been shown to segregate in a Mendelian fashion, they have been used in the development of genetic linkage maps. Such maps are made up of closely-spaced DNA markers and within these maps close linkage were found between molecular marker and a trait of interest in crop improvement (Newbury and Ford-Lloyd 1993). RAPDs have also been used in DNA fingerprinting which aids in identification and characterization of crop species and in the identification of hybrids.

RAPDs are relatively fast and easy to produce and do not require radioactivity. However they have a number of limitations. Most RAPDs are dominant, i.e. it is difficult to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. Co-dominant RAPD markers are rare (Newbury and Ford-Lloyd 1993). Also there is lack of cross-transferability and mismatches between the primer and the template may result in total absence of PCR product as well as in a merely decreased amount of the product.

RAPD markers have been developed in tomato, sorghum, finger millet, barley and rice for marker assisted selection (Mohan, Nair et al. 1997; Das and Misra 2010). Similar to RFLPs, RAPDs have also been put to only limited use in wheat, owing to its low level of polymorphism and the lack of reproducibility of results (Gupta, Varshney et al. 1999).

1.3.3 Simple sequence repeats

Polymorphic loci present in nuclear DNA that consist of repeating units of 1-6 base pairs are called microsatellites, simple sequence repeats (SSR), short tandem repeats (STR), simple sequence repeats polymorphism (SSRP), sequence-tagged microsatellite sites (STMS), or simple sequence length polymorphisms (SSLP). These markers are highly variable and ubiquitous within the eukaryotic genome and can be analyzed through PCR technology. Variations in the number of tandem repeat sequences of nucleotides at a SSR locus among different genotypes provide the basis for polymorphism, which can be detected using primers flanking the specific microsatellite loci. SSR markers thus reveal

polymorphisms due to variation in length of microsatellites at specific individual loci (Jain and Brar 2010).

Microsatellites are widely distributed in eukaryotic species (Gupta, Balyan et al. 1996). New microsatellites can be cloned directly from total genomic libraries or libraries enriched for specific SSRs. Alternatively, known SSRs are described in databases like EMBL and GeneBank. Primers may then be designed for the known flanking sequences either by manual inspection or with the aid of computer programs.

So far, SSRs have been used for marker assisted selection (MAS), genome mapping, cultivar identification, estimation of genetic relatedness and germplasm (Collard and Mackill 2008). SSRs are useful markers as they represent a single locus, and are co-dominant and multi-allelic. They do not require radioactivity and multiplex reactions can be run to speed up the assay, where the products have non-overlapping size ranges (Henry 2001). The greatest disadvantage is the initial cost in the identification and sequencing of loci. However, once they are established, SSR markers become a highly informative source for germplasm management and mapping.

Microsatellites have been widely studied in *S. cervisiae*, *P. sativum*, *A. thaliana*, *Z. mays*, *O. sativa* and many other species (Gupta, Balyan et al. 1996). Microsatellites were reported in hexaploid wheat beginning 1995. These markers were genome specific and displayed a high level of variation. In 1998, a detailed genetic map of 279 SSRs and another map containing 53 SSRs were prepared in

bread wheat (Gupta, Varshney et al. 1999). The available genetic map of finger millet includes 82 SSR markers (Dida, Ramakrishnan et al. 2007).

1.3.4 Single nucleotide polymorphism

SNP alleles differ by a single base. This marker system can be detected either by gel-based or non-gel based assays. Characterization of SNPs in humans suggested that one SNP can be found on average every 1 kbp of sequence and the potential of SNP markers is clearly demonstrated in human genome analysis (Ganal, Altmann et al. 2009). SNPs are also abundant in plant genomes, although they are not yet as widely used as SSRs. The main reason is because of the cost involved in developing SNPs.

Identification of SNPs within a genetic locus varies with different crops. SNPs can be identified based on EST sequence data, array analyses, amplicon re-sequencing, next generation sequencing technologies and from sequenced genomes (Ganal, Altmann et al. 2009). Though their abundance makes them attractive, it is difficult to work with SNPs when there is little information available about the species under consideration (Jain and Brar 2010). SNPs are used in mapping, marker-assisted breeding and map-based cloning (Semagn, Bjørnstad et al. 2006).

SNPs are being identified important crop plants like tomato, maize, pepper and others (Ganal, Altmann et al. 2009). A recent study aimed at mapping QTL for heat tolerance at flowering stage in rice using SNP markers and showed that 280 out of 384 markers (72.9%) showed polymorphism between the two selected lines of rice IR 64 and N 22 (Ye, Argayoso et al. 2012).

1.4 Uses of markers in germplasm collection

Molecular markers are being used as powerful tools in managing plant germplasm collections, both *in situ* and *ex situ*. With the advent of molecular techniques, efficiency and amount of information generated has increased with a decrease in cost and time involved. Genetic diversity analysis and fingerprinting are two distinct, but related, techniques. The former gives the relative measure of the genetic distances between genotypes in a defined set using pre-selected number of markers. The latter is an absolute measure of the genetic makeup of an individual line, and must be unique to that line in order to distinguish it from all others (Henry 2001).

The following imitated list (Warburton and Hoisington 2001) outlines the proposed capacities of genetic markers in plant genetic resource management:

1. To search for correlations of traits and markers in related individuals without mapping.
2. To narrow the search for new alleles in loci of interest.
3. To verify pedigrees.
4. To assign lines and populations to heterotic groups.
5. To choose parents for mapping, MAS and backcrossing.
6. To monitor changes in allele frequencies in populations.
7. To study evolutionary history of wild relatives.

As an example, molecular markers have been used to study the genetic diversity of wild populations of tea tree (*Melaleuca alternifolia*). This study was

based on an enriched library of SSRs, from which 93 of 139 primers exhibited polymorphism among five plants (Henry 2001). Genetic diversity in rice (*O. sativa*) was also studied by a group using four molecular marker systems and discriminating their effectiveness. They examined the diversity of 42 accessions using 37 isozymes, 41 AFLP, 40 RAPD and 38 ISSR (Virk, Zhu et al. 2000). It was shown that there was essentially no difference between the marker techniques used for determination of polymorphism. However, there needed to be caution over the choice of technique in determining relationships between these groups using cluster analysis. A number of studies have been done on many crop plants like wheat, barley, sorghum, etc. using a number of molecular markers (Jain and Brar 2010).

1.5 Genetic diversity of finger millet

The ICRISAT gene bank holds 5,949 finger millet accessions from 24 countries. Efforts have been made to characterize this population on basis of yield, calcium content, and protein content. One of the preliminary studies addressed the genetic similarity between *E. coracana*, *E. indica*, *E. tristachya*, *E. compressa* and *E. floccifolia*, to understand the diversity that exists within the world collection of finger millet (Salimath, Oliveira et al. 1995). The use of 8 probe-3 enzyme RFLP combination, 18 RAPD primers and 6 inter simple sequence repeat amplification (ISSR) primers revealed 14, 10 and 26% polymorphism respectively. They indicated that *E. coracana*, *E. indica* and *E.tristachya* shared most markers thus forming a close assemblage within the *Eleusine*.

A major step was taken in 2006 to create a core subset of finger millet germplasm from the entire global collection of 5940 accessions held in ICRISAT, Patancheru, India (Upadhyaya, Gowda et al. 2006). The objective behind this study was to boost the utilization and productivity of finger millet germplasm to breed superior varieties. Characterization took place in the research farms of ICRISAT, Patancheru from 1974-2003. Data was recorded on eight qualitative traits (plant pigmentation, growth habitat, inflorescence, compactness, glume prominence, seed color, lodging, senescence and overall disease free) and 14 quantitative traits (flowering, basal tiller number, plant height, number of culm branches, flag leaf blade length, flag leaf blade width, flag leaf sheath length, peduncle length, panicle exertion, inflorescence length, inflorescence width, longest finger length, longest finger width and number of panicle branches). The entire collection was grouped into 104 clusters and a core subset of 622 accessions was formed based on the 14 quantitative traits.

With advances in breeding, the need arose for the identification of diverse lines of finger millet for hybridizing to produce better yielding varieties of finger millet. Thus in 2007, a study was conducted to identify the finger millet accessions with the widest genetic distance by assessing the extent of variation in different accessions at molecular level (Babu, Senthil et al. 2007). This study employed 50 RAPD markers to assess 32 accessions obtained from different states of India. 479 of the 529 loci generated using the RAPD markers exhibited polymorphism, which aided in the differentiation of the accessions.

At the molecular level, finger millet germplasm still largely remains uncharacterized. Efforts have been made to use various molecular marker systems like RAPD, SSR and cytochrome P_{450} to analyze the genetic diversity present in different accessions of finger millet (Das and Misra 2010; Kumari and Pande 2010; Panwar, Nath et al. 2010; Panwar, Saini et al. 2010; Kumar, Sharma et al. 2012). Of the various marker systems employed in assessing the genetic diversity of finger millet germplasm, SSRs have been shown to be the most effective (Panwar, Nath et al. 2010). While diversity studies were being conducted at a large scale, mapping efforts in finger millet is still at infancy. The first partial map of finger millet was produced in 1988. Later mapping efforts led to the development of a larger *E. coracana* genetic map, with the aid of RFLP, AFLP, EST and SSR markers, where 379 loci were grouped into 18 linkage groups (Dida, Ramakrishnan et al. 2007).

1.6 Objectives

Alleviating poverty and malnutrition in agro-biodiversity hotspots is a project designed by the scientists at MSSRF, India and University of Alberta, Canada, with the objective to enhance food, nutritional security and income of rural poor in India. The aim of the present study is to aid this project through plant genetics. Because of governmental limitations on access to germplasm from Indian seed banks, this project analyses a collection of 83 finger millet accessions of diverse geographic origin distributed by ICRISAT.

The first component of the study is a physiological and gene expression assessment of a finger millet germplasm collection with respect to its drought stress responses. Physiological measurements will include measurement of relative water content, estimation of proline, chlorophyll, ion leakage and anthocyanin, and observation of plant activity in terms of stomatal conductance, photosynthetic rate and transpiration rate. The second component is designed to expand the genomic resources available for finger millet, including further development of markers that can be used to assess genetic diversity of finger millet germplasm collections.

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Chapter 2 – Physiological responses of finger millet (*Eleusine coracana* (L.) Gaertn.) to water deficit

2.1 Introduction

Finger millet (*Eleusine coracana* (L.) Gaertn.) has a reputation as a hardy crop favored by subsistence farmers for its stress tolerance and its stability in storage (Singh and Raghuvanshi 2012). It has particular value in nutritional security as a rich source of calcium and essential amino acids like methionine and tryptophan, which are lacking in some other cereals (Mbithi-Mwikya, Ooghe et al. 2000). Finger millets are also rich in specific flavonoids and free phenolic acids, which may provide health benefits by acting as antioxidants and inhibitors of enzymes including alpha-amylase (Shobana, Harsha et al. 2010; Shahidi and Chandrasekara 2013). Studies in humans have shown that finger millet consumption can lower blood glucose levels in diabetics and protect against hyperglycemia (Kumari and Sumathi 2002). The inhibition of metabolic enzymes, along with the grain's high fiber content (Shobana, Sreerama et al. 2009), may explain the prolonged sense of satiety that finger millet is reputed to provide.

Amongst various environmental constraints, drought is very prominent and affects the productivity and utilization of finger millet (Obilana 2010). Finger millet cultivation falls under the savannah agro-ecosystem which is characterized by frequent droughts (Rockström 2003). With a striking nutritional profile (Saleh, Zhang et al. 2013), finger millet became an important subject to study the stress tolerance from a physiological and molecular perspective. Several studies have

reported that finger millet is a stress-hardy crop that is able to withstand long durations of water deficiency. One recent study applied field-level drought stress to finger millet and evaluated gene expression by e-northern analysis RT-PCR and in leaf tissues (Parvathi, Nataraja et al. 2012). The outcome was identification of several stress responsive genes including metallothionein, farnesylated protein ATFP6, protein phosphatase 2A, RISBZ4 and farnesyl pyrophosphate synthase which might have crucial roles in imparting hardiness to finger millet (Parvathi, Nataraja et al. 2012).

Another study surveyed antioxidant potential of five diverse accessions of finger millet and provided evidence that finger millet responds to drought stress by enhancing its antioxidative capacity and APX:SOD (Bhatt, Negi et al. 2011; Bhatt, Saxena et al. 2013). To further assess the range and type responses of finger millet to stress, we characterized the physiological responses of diverse accessions of finger millet following water withholding. Our objective was to identify germplasm with distinct stress responses and provide a basis for future comparative and mechanistic studies of stress tolerance in this species.

2.2 Materials and Methods

2.2.1 Plant growth, stress treatments, and sampling

Seeds were obtained from ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India). Seeds were sown in Sunshine Mix 4 (Sun Gro Horticulture, USA) ~200 g/pot, planted in round pots (9 cm height, 10 cm diameter at the top), to a depth of approximately 1 cm, at a

density of 3-5 seeds per pot. After germination, seedlings were thinned to one per pot. Plants were grown in environmental growth chambers at 29°C with ~42% relative humidity, and a photosynthetic photon flux (PPF) of 110 ± 10 μmole m^{-2} s^{-1} supplied by high output fluorescent bulbs on a 12 h light/12 h dark cycle.

All plants, in both the control and treatment groups, were watered regularly for up to 26 days after seeding (DAS). Plants were fertilized with 100 ppm nitrogen 14 DAS with Plant-Prod 20-20-20 water soluble fertilizer (Plant Prod, Ontario, Canada). Water deficit stress was initiated 26 DAS and progressive drought (pDr) was imparted for 6 days by withholding water supply. Control plants were watered every day until the soil was completely saturated. Fully grown young leaf blades were sampled from control and stressed plants. Sampling was done after 6 days of imposition of stress. Experimental design included three biological replicates (temporally and spatially separated) which were set up in three different growth chambers and each biological replicate consisted of three technical replicates.

2.2.2 Physiological and biochemical assays

Relative water content (RWC) was determined for each accession (both control and treatment) using three technical replicates. Leaf blades from three plants of each accession were pooled together and fresh weight (FW) was measured. Leaves were then incubated in a deionized water-filled Petri plate for at least 10 h until they reached full turgidity. The leaf blades were then surface dried by paper towel and re-weighed for turgid weight (TW). Samples were then oven-dried at 70°C for 72 h and their dry weight (DW) was recorded. RWC was

calculated as RWC% = [(FW-DW)/(TW-DW)]*100 (Barrs and Weatherley 1962).

Proline content in fully developed young leaf blades was determined by the ninhydrin method (Bates, Waldren et al. 1973). Fresh leaf tissue (~0.5 g) was homogenized in 10 mL of sulfo-salicyclic acid (3% w/v) on ice. The resulting filtrate (2 mL), acid ninhydrin (2 mL) and glacial acetic acid (2 mL) were mixed and incubated at 100°C for 1 h. The mixture was cooled to room temperature and 2 mL toluene was added and vortexed for 5 s. The upper aqueous layer containing the chromophore was removed and measured by spectrophotometer (PowerWave HT Microplate Spectrophotometer, BioTek) at 520nm using L-Proline as a standard.

Total anthocyanin content in finger millet was determined as previously described (Abdel-Aal and Hucl 1999). Frozen leaf samples (~3 g) were ground to a fine powder with liquid nitrogen. Acidified ethanol (24 mL; ethanol and HCl 1 N, 85:15 v/v) was added to the ground tissue and transferred to a 50 mL centrifuge tube. This solution was mixed and the pH was adjusted to 1.0 with 4 N HCl. The solution was then agitated for 15 minutes at 220 rpm and pH was re-adjusted to 1, if required. Tubes were centrifuged at 27,200 X *g* for 15 minutes. Supernatant was poured into a 50 mL flask and made up to volume with acidified ethanol. Absorbance was measured by spectrophotometer (PowerWave HT Microplate Spectrophotometer, BioTek) at 535 nm using cyanidin-3-glucoside as a standard.

Electrolyte leakage (EL) was measured using established protocols (Lutts, Kinet et al. 1996) with a few modifications. Fresh leaves (~0.3 g) were washed with de-ionized water and incubated in tubes containing 15 mL de-ionized water for 2 h at room temperature (21-24°C). Electrical conductivity (EC) of water (EL_1) was measured after 2 h using an electrical conductivity meter (Oakton CON 11, Cole Parmer, Canada). Cell membranes were disrupted by heating the samples at 120°C for 30 minutes and the final conductivity (EL_2) was measured after equilibration at 25°C. Membrane stability was presented as percent electrolyte leakage = $[(EL_1/EL_2)*100]$.

Net photosynthetic rate (P_n; μmol m^{-2} s^{-1}), transpiration rate (E; mmol m^{-2} s^{-1}), stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}) and water use efficiency (WUE; %) were monitored by means of an Infra Red Gas Analyzer (IRGA) (Model LI 6400, LI-COR® Inc, Nebraska, USA).

Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC) content were analyzed by the acetone method (Shabala, Shabala et al. 1998) and total carotenoid (C_{x+c}) concentration was also determined (Lichtenthaler 1987). Leaf discs (~0.1 g) from fully developed young leaves were collected in a 15 mL tube containing 10 ml of acetone (100%). The tubes were covered and stored at 4°C for 48 h. Chl_a, Chl_b and C_{x+c} concentrations were measured using a spectrophotometer (PowerWave HT Microplate Spectrophotometer, BioTek) at 662 nm, 644 nm and 470 nm respectively using 100% acetone as blank.

2.2.3 Statistical analysis

All physiological variables (RWC, ion leakage, proline content, anthocyanin content, chlorophyll, carotenoids and photosynthetic parameters) were analyzed as a split-plot analysis of variance using PROC MIXED (SAS Institute, Cary, NC) with treatment (drought and control) as the main plot, variety (IE 4757, IE 5091, IE 6537, IE 7079 and IE 2797) as the sub plot and interaction, and replicate (n=3) as the random effect. Differences in least squares means were obtained using an LSD test and significance declared at $P<0.05$. The data are presented as least squares means and standard errors of means. The correlations between water holding capacity and selected variables were obtained using the PROC CORR procedure of SAS.

2.3 Results

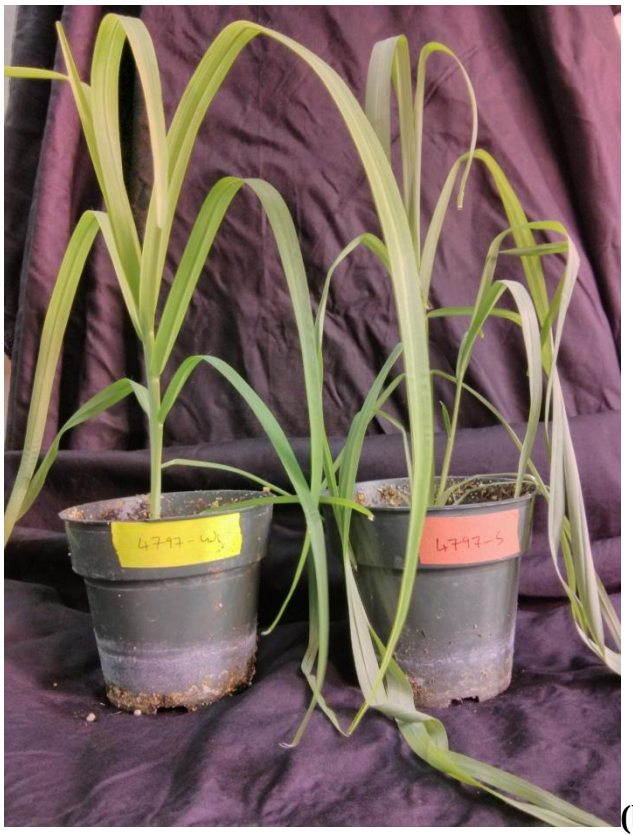
2.3.1 RWC

We measured the effects of withholding water on five accessions of finger millet (Table 2.1). These accessions were selected in a preliminary screen of a geographically diverse mini-core collection (Upadhyaya, Sarma et al. 2010) to represent a range of stress responses (Appendix 2.7.1). After 6 days of water withholding, wilting was visible in the treated lines (Fig. 2.1) and statistically significant decreases in RWC were observed in four of the five accessions (Fig. 2.2). The greatest decrease in RWC was experienced by IE 7079 (2.2 fold, $p<0.01$). On the other hand, no measurable differences were recorded in IE 4757. Well-watered controls were analyzed in parallel.

Table 2.1: List of five accessions of finger millet used in the physiological study. Details of accession lines with their corresponding alternate accession identifier and geographic origin.

Accession number	**Country**	**Alternate accession identifier**
IE4757	India	Ragi
IE5091	Zimbabwe	SDFM 313
IE6537	Nigeria	-
IE7079	Kenya	SDFM 1987; 18816
IE4797	Maldives	KLM 1868

(a)



(b)


(c)

(d)


(e)

Figure 2.1: Wilting observed in the five genotypes. (a) IE 4757; (b) IE 4797; (c) IE 5091; (d) IE 6537; (e) IE 7079, when subjected to 6 days of water deficit (yellow label: control/well-watered; red label: treatment/water-deficit)

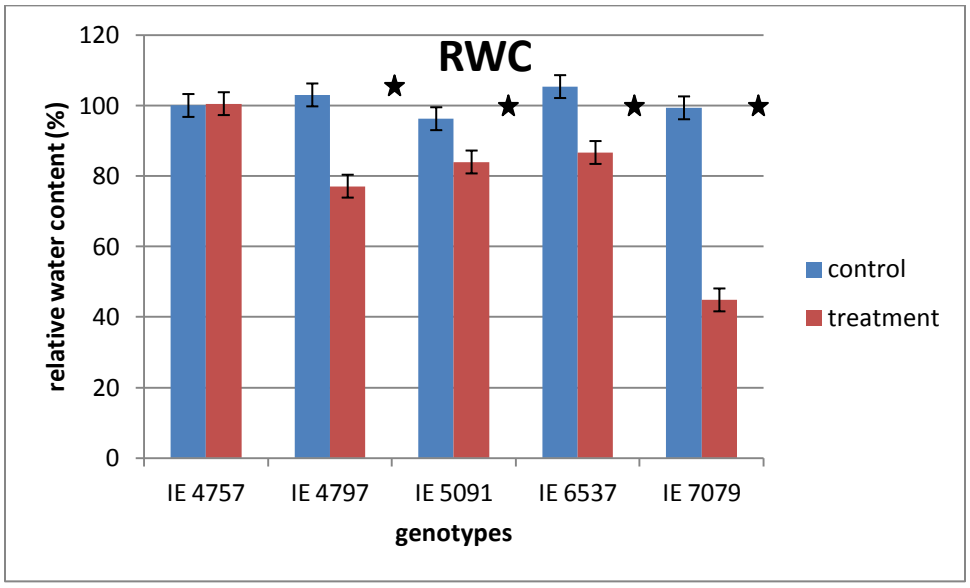


Figure 2.2: Effect of water stress on relative water content of all five accessions of finger millet. Water stress was applied for 6 days by arresting water supply to treated plants. RWC was determined as previously described (Barrs and Weatherley 1962). Each value represents mean of three independent experiments (±SD)

2.3.2 Proline content

Proline concentration increased significantly in four accessions except IE 5091. The most significant increase (1.4 fold, $p < 0.01$) in the stressed leaves as compared to well-watered controls was observed in IE 7079 (Fig. 2.3). IE 7079 showed the highest proline concentration of 0.014 μmol g^{-1} and IE 5091 showed the lowest proline concentration of 0.010 μmol g^{-1} following treatment.

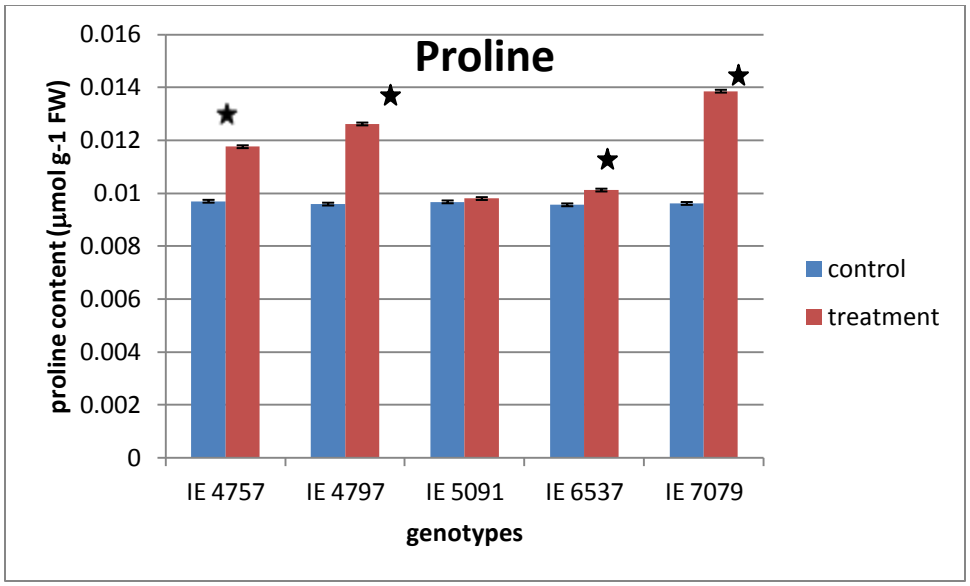


Figure 2.3: Proline accumulation in five accessions of finger millet when subjected to water stress. Proline content (µmol g^{-1} FW) was determined in both well-watered and water-deficit plants as previously described (Bates, Waldren et al. 1973), after 6 days of water stress. Each value represents mean of three independent experiments (±SD)

2.3.3 Anthocyanin content

Anthocyanins are water-soluble pigments that are found in most plant tissues and contribute to environmental stress resistance and photoprotection (Chalker-Scott 1999). Following imposition of water stress, anthocyanin content in four genotypes increased significantly (Fig. 2.4). Accession IE 7079 had the highest concentration with 84.15 mg kg^{-1} and IE 6537 had the lowest accumulation with 61.16 mg kg^{-1}. A maximum increase of 1.6 fold ($p<0.01$) in anthocyanin content was recorded in IE 4757, closely followed by IE 4797 and IE 7079 with 1.5 fold ($p<0.01$) and 1.4 fold ($p<0.01$) increases, respectively (Fig. 2.4).

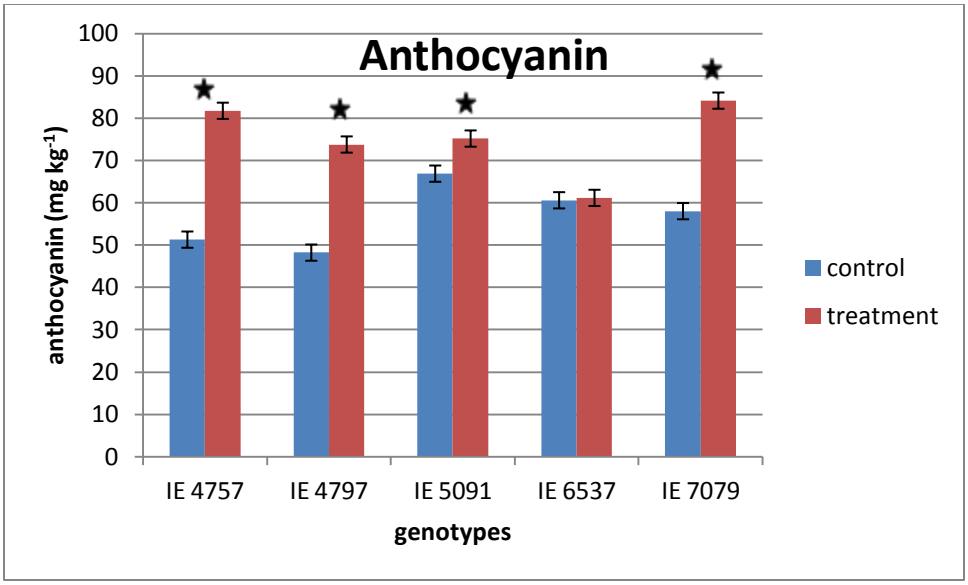


Figure 2.4: Anthocyanin accumulation in five accessions of finger millet when subjected to water stress. Following 6 days of water stress, anthocyanin content (mg kg^{-1}) was determined in both well-watered and water-deficit plants as previously described (Abdel-Aal and Hucl 1999). Each value represents mean of three independent experiments (±SD)

2.3.4 Electrolyte leakage

Cell membrane stability under water deficit conditions was assessed by measuring the ion leakage from both control and treated plants (Fig. 2.5). Accessions IE 4797 ($p<0.01$), IE 6537 ($p<0.01$) and IE 7079 ($p<0.01$) showed a significant increase in ion leakage (3 fold, 7.5 fold and 5.1 fold increase, respectively). Following treatment, IE 6537 showed the most ion leakage at 18.2% and IE 4757 showed the least ion leakage at 2.3%. The other two accessions, namely IE 4757 and IE 5091 showed no measureable change in ion leakage.

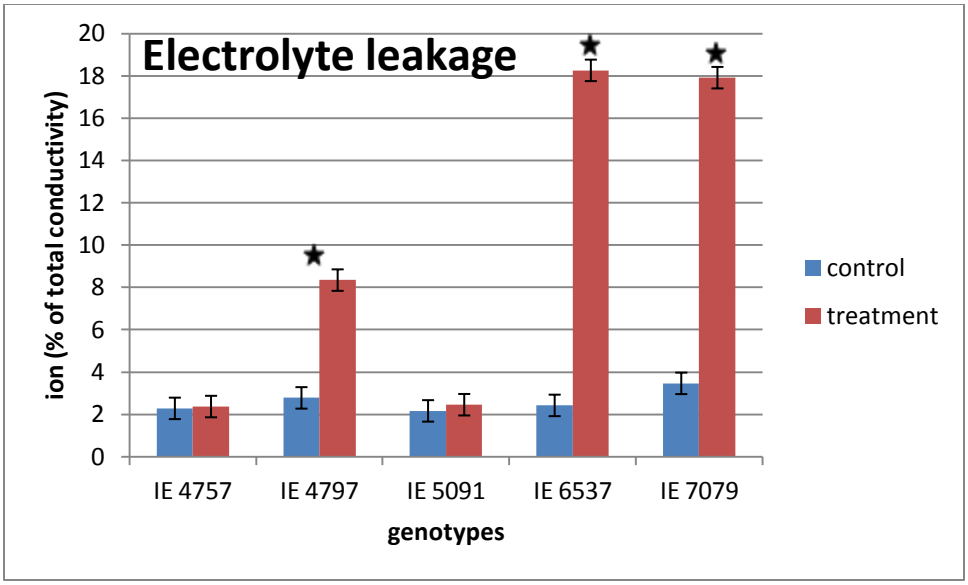
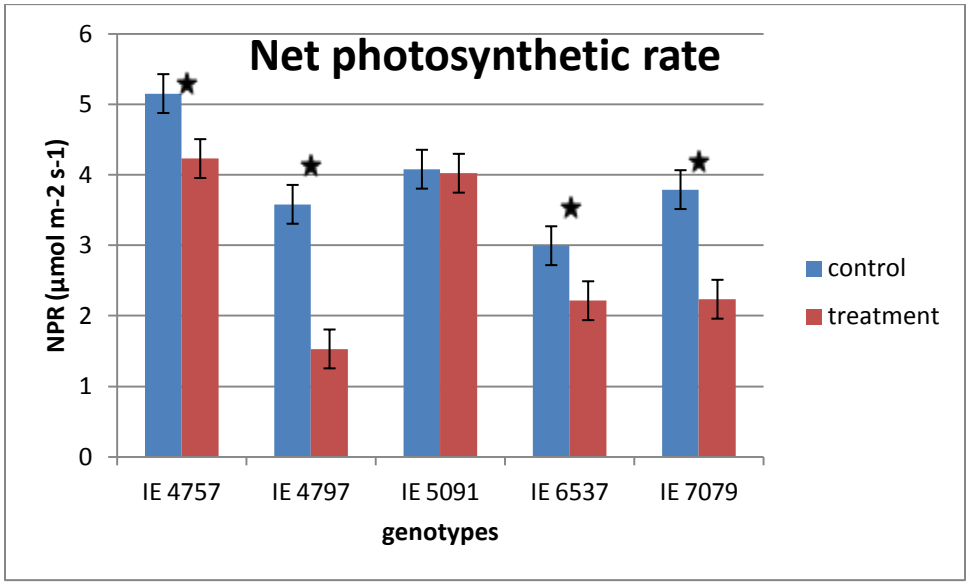


Figure 2.5: Membrane damage caused to the five accessions of finger millet. Membrane damage was quantified as a measure of ion leakage and was measured using a electrical conductivity meter (Lutts, Kinet et al. 1996). Each value represents mean of three independent experiments (±SD)

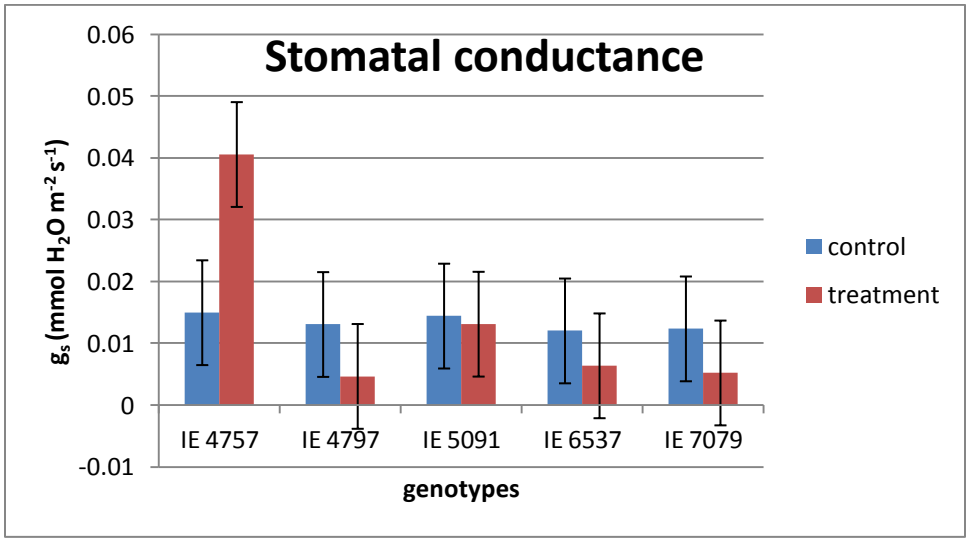
2.3.5 Photosynthetic activity

Following water stress treatment, accessions IE 5091 and IE 6537 maintained their pre-stress photosynthetic activity (net photosynthetic rate (P_n)), whereas P_n of IE 4757 ($p=0.03$), IE 4797 ($p<0.01$) and IE 7079 ($p<0.01$) declined significantly (0.8, 0.4 and 0.6 fold, respectively) compared to their respective controls (Fig. 2.6a). Under stress conditions, IE 4757 had the highest P_n (4.2 µmol m^{-2} s^{-1}) whereas IE 4797 recorded the lowest P_n (1.5 µmol m^{-2} s^{-1}). After water withholding, no significant difference was observed for g_s in the five accessions (Fig. 2.6b). A general trend of declined transpiration rate (E) in five genotypes was observed under stress. All five lines were significantly different ($p<0.01$) from their respective controls. Line IE 4757 recorded the highest E under stress,

at 0.92 mmol m^{-2} s^{-1}, and IE 4797 recorded the lowest E with 0.26 mmol m^{-2} s^{-1} (Fig. 2.6c). The maximum decrease of E following water withholding was observed in IE 4797 (3.5 fold) and the lowest decline was observed in IE 4757 (1.2 fold) (Fig. 2.6c). A significant increase in water use efficiency (WUE) was observed in IE 6537 (p=0.02) and IE 7079 (p=0.02). IE 6537 recorded the highest WUE at 707% and IE 4757 showed the lowest WUE at 455%, following water stress (Fig. 2.6d).



(a)



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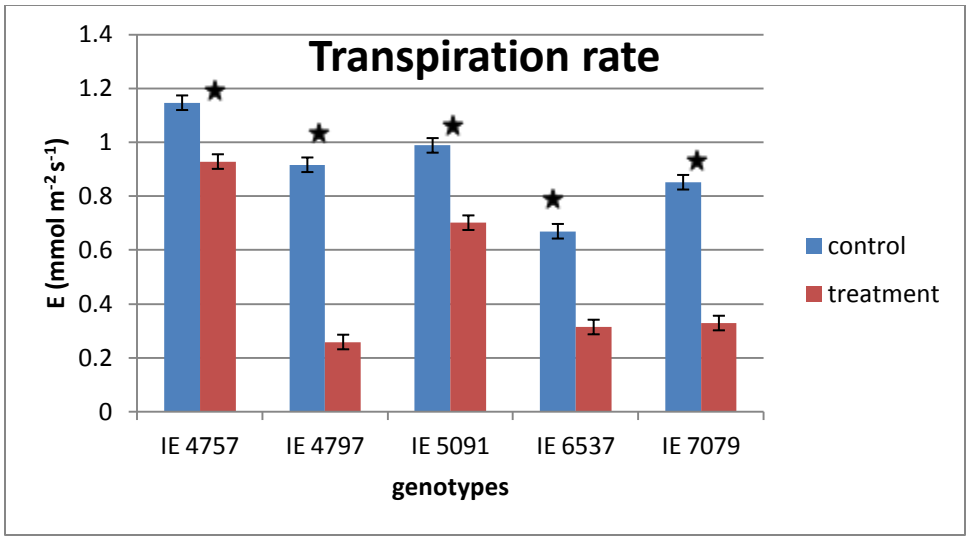

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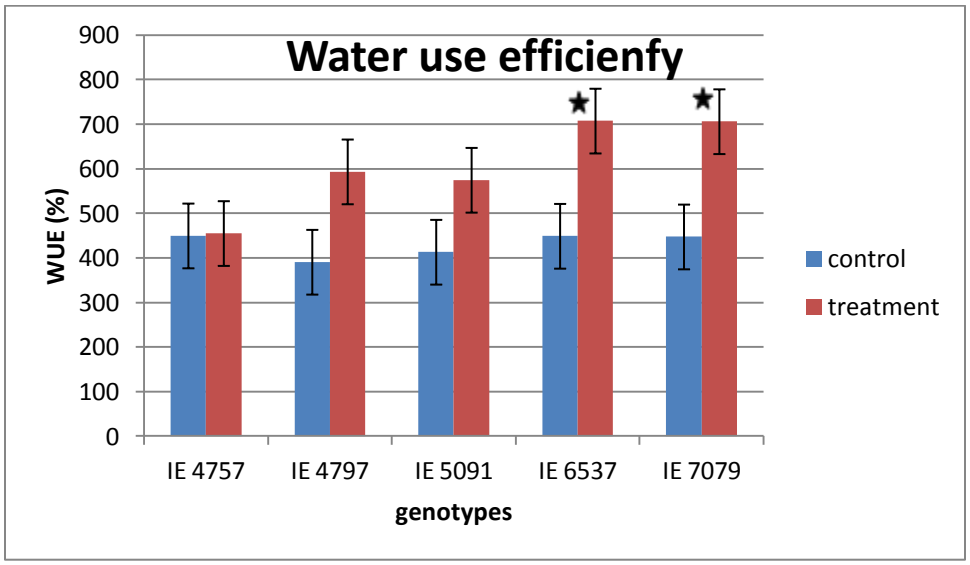
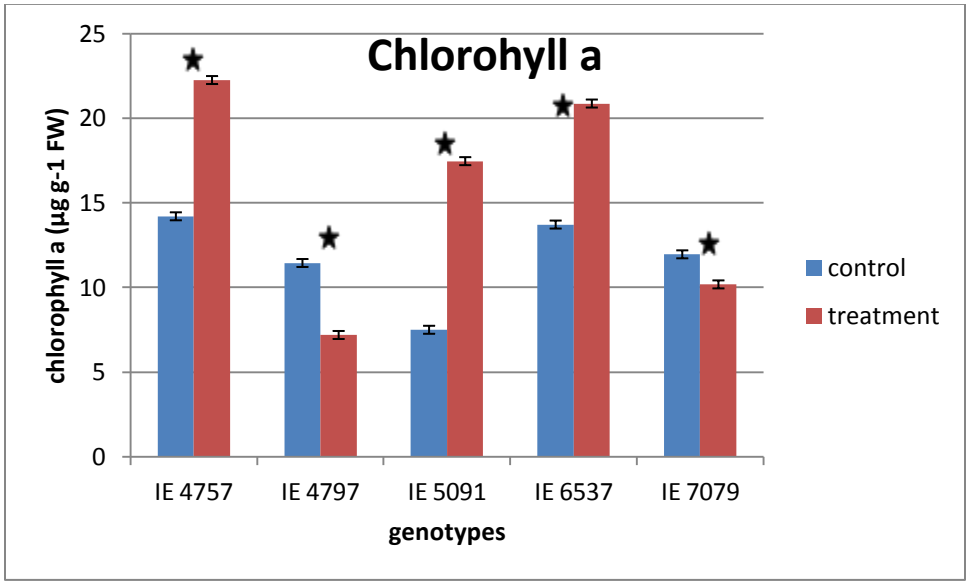


(d)

Figure 2.6: Specific photosynthetic parameters recorded in the five accessions of finger millet following water stress. (a) Net photosynthetic rate (µmol m^{-2} s^{-1}); (b) stomatal conductance (mmol H_2O m^{-2} s^{-1}); (c) transpiration rate (mmol m^{-2} s^{-1}); (d) water use efficiency (%) in the five different accessions of finger millet, when subjected to 6 days of water stress. All observations were made using an infra-red gas exchange analyzer (Model LI 6400, LI-COR® Inc, Nebraska, USA). Each value represents mean of three independent experiments (±SD)

2.3.6 Chlorophyll and carotenoid content

A significant difference in total chlorophyll content (TC) was observed in all five accessions following stress (Fig. 2.7c). IE 4757, IE 4797 and IE 6537 showed a significant increase following stress, whereas, IE 4797 and IE 7079 showed a significant decline. The highest TC under stressed conditions was recorded in IE 4757, which contained 41.7 µg g^{-1} FW and the lowest in IE 7079, which contained 10.30 µg g^{-1} FW. IE 5091 showed the greatest increase in TC (3.3 fold) and IE 4797 showed the greatest decline (1.4 fold) in TC under stressed condition. The two major types of chlorophyll: Chl_a and Chl_b were also measured. A significant increase in Chl_a was observed in three lines: IE 4757, IE 5091, and IE 6537 ($p<0.01$) and a significant decrease ($p<0.01$) of Chl_a in the stressed plant of IE 4797 and IE 7079 (Fig. 2.7a). In IE 7079, a significant decline (1.1 fold) in Chl_a was accompanied by a significant decline (2.2 fold) in Chl_b (Fig. 2.7b)). Total carotenoids increased significantly in IE 5091, IE 6537, and IE 7079 ($p<0.01$) and decreased in IE 4757 and IE 4797 ($p<0.01$), under stress (Fig. 2.7d).



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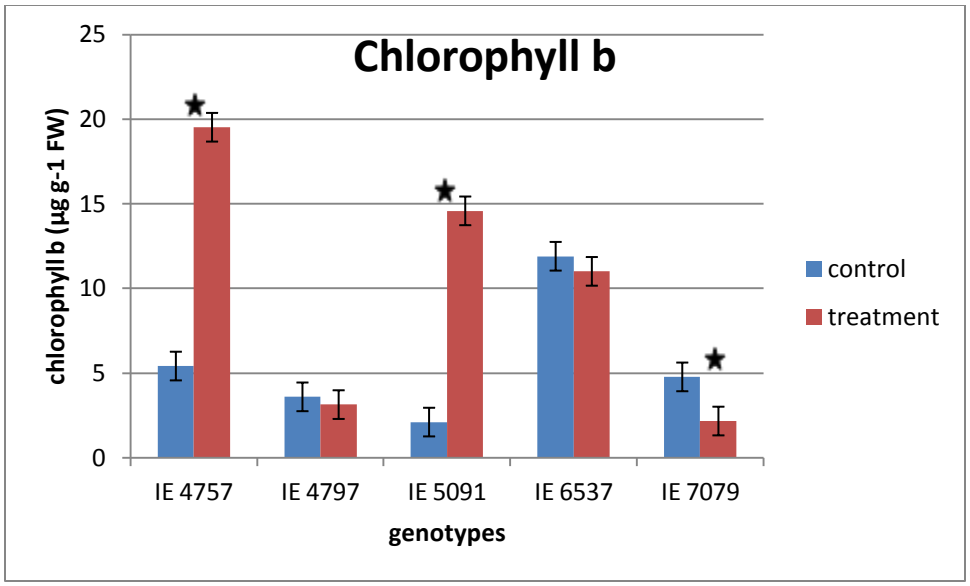
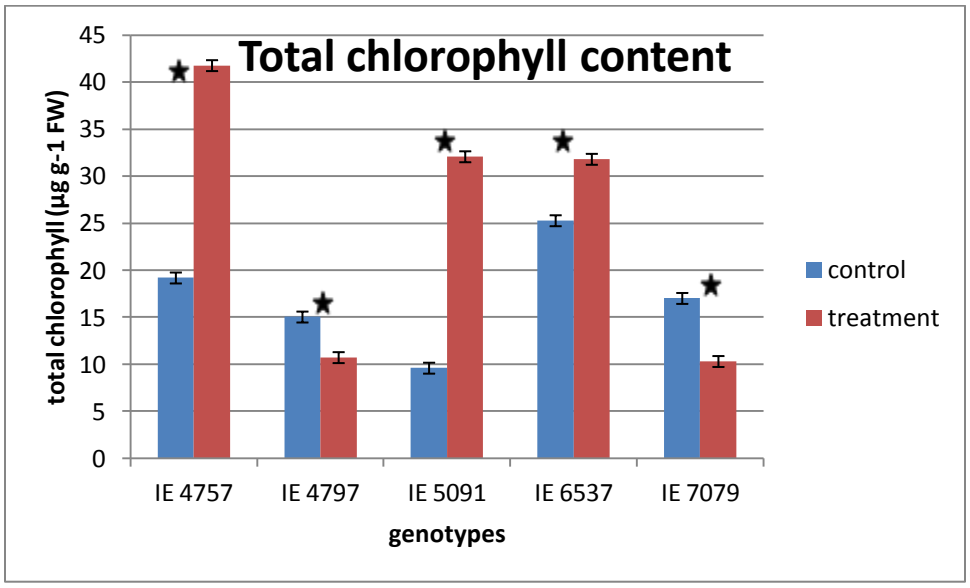
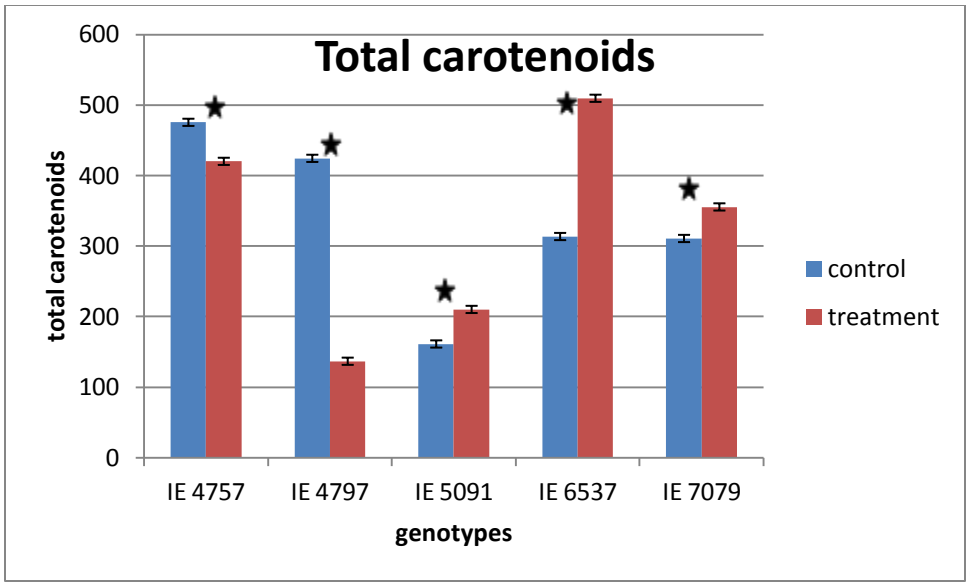

Chlorophyll b
chlorophyll b (µg g-1 FW)
25
20
15
10
5
0
IE 4757
IE 4797
IE 5091
IE 6537
IE 7079
genotypes
control
treatment

(b)


Total chlorophyll content
total chlorophyll (µg g-1 FW)
45
40
35
30
25
20
15
10
5
0
IE 4757
IE 4797
IE 5091
IE 6537
IE 7079
genotypes
control
treatment

(c)



(d)

Figure 2.7: Chlorophyll and carotenoid contents in the five accessions of finger millet. (a) Chlorophyll a (Chl_a); (b) Chlorophyll b (Chl_b); (c) total chlorophyll content (TC); (d) total carotenoids (C_{x+c}) contents measured in the fully matured young leaves of different finger millet accessions grown under water deficit conditions and measured using a spectrophotometer (PowerWave HT Microplate Spectrophotometer, BioTek). Each value represents mean of three independent experiments (±SD)

2.4 Discussion

2.4.1 RWC, proline content, anthocyanin, and ion leakage

RWC provides a measure of plant water status and was expected to decrease in the stressed lines. A significant impact ($p<0.05$, determined by PROC MIXED: SAS Institute, Cary, NC) of water stress was observed in all four accessions except IE 4757 (Fig. 2.2). This may suggest that IE 4757 can perceive and respond to very small changes in RWC.

Proline is an osmolyte and serves as an indicator of drought stress responses. Proline concentration has been used with other physiological parameters to understand the physiological responses of plants to drought (Bhatt, Negi et al. 2011; Parvathi, Nataraja et al. 2012). In our study, proline accumulation was observed in all the five accessions of finger millet under water stress. However there was no significant accumulation of proline in IE 5091 under stress (Fig. 2.3) even though the accession responded by a change in its water status (Fig. 2.2). IE 5091 accumulated 0.01 μmol g^{-1} FW of proline at 84% RWC, whereas the other four accessions accumulated more than half as much as IE 5091 under similar conditions. Drought induced proline accumulation has been observed in many plant species, namely rice, maize, and finger millet and has been associated with adaptation to drought stress (Valentovic, Luxova et al. 2006; Cha-Um, Yooyongwech et al. 2010; Bhatt, Negi et al. 2011). Studies on finger millet have shown that it accumulates proline during water stress, which in turn stabilizes protein synthesis (Kandpal and Rao 1985).

Under water stress, four accessions of finger millet accumulate significant amount of anthocyanins, but IE 6537 did not (Fig. 2.4). Anthocyanins are water-soluble pigments that are found in all plant tissues and contribute resistance to environmental stresses. A review outlines the importance of anthocyanins in plants to stresses induced by visible and UVB radiation, drought, and cold (Chalker-Scott 1999). Increased accumulation of anthocyanins is known to shield dehydrated plants from photo-inhibition and offer protection to leaves without significantly affecting photosynthesis (Steyn, Wand et al. 2002). Thus a higher

accumulation of anthocyanin in all the four lines (Fig. 2.4), under water withholding can be correlated to better stress tolerance characteristics. Our results were consistent with a similar study that was conducted on *Arabidopsis thaliana* ecotype Columbia plants where increased accumulation of metabolites (proline, soluble sugars and anthocyanin) were shown to play important roles in acclimating to drought stress (Sperdouli and Moustakas 2012). A 1.9 fold increase in anthocyanin accumulation was reported in *Arabidopsis thaliana* plants when subjected to 6 days of water stress (Sperdouli and Moustakas 2012), which is comparable to an average of 1.3 fold increase in our studies.

Cell membranes are early targets of many plant stresses and are subject to defects including an increase in permeability, stability and integrity under water stress (Bajji, Kinet et al. 2002). Thus, the ability of a cell membrane to control the movement of ions in and out of the cell serves as an indicator of physiological status. Maximum drought induced membrane damage was recorded in IE 6537 that showed a 7.5 fold increase, which was closely followed by IE 7079 and IE 4797 with a 5.1 fold increase and a 3 fold increase, respectively (Fig. 2.5). Membrane damage under water stress can be attributed to peroxidation of membrane lipids (Bhatt, Negi et al. 2011). Thus a lower electrolyte leakage in IE 4757 and IE 5091 indicates that these accessions experience less membrane damage than the other three accessions under water limiting conditions. Consistent with previous studies on maize (Quan, Shang et al. 2004) and finger millet (Bhatt, Negi et al. 2011), our results have shown an increase in ion leakage in three accessions under water stress. The significant increases in accession IE

7079, IE 6537, and IE 4797 indicates that these varieties are susceptible to membrane damage and cannot withstand water stress when compared to the other two accession lines.

2.4.2 Photosynthetic parameters

Decreasing photosynthetic rate (P_n) is a common response of plants to water deficit stress. This response could be attributed to either stomatal closure or metabolic impairment (Costa França, Pham Thi et al. 2000). Leaf water potential and stomatal conductance (g_s) are correlated under drought, largely as a result of an attempt to conserve available water. Lower P_n can also be attributed to cumulative, non-stomatal, and biochemical effects of stress. For instance, increased accumulation of ions is known to reduce CO_2 assimilation as a result of ion toxicity (Djanaguiraman, Sheeba et al. 2006). A third parameter we measured is transpiration (E). Transpiration is the process of water loss from the plant in the form of water vapor from leaves and other aerial parts. Under water stress, transpiration is known to decrease as a reflex to drought stimuli (Souza, Machado et al. 2004). Finally, water use efficiency (WUE), describes the total dry matter produced per unit of water transpired (Cabuslay, Ito et al. 2002). It is often represented as a ratio of photosynthetic rate and transpiration rate (Blum 2005). Reduced E can be associated with higher WUE (Tolk and Howell 2003).

Measurement of photosynthetic parameters (P_n, g_s, and E) in our study helped us understand the response of a plant to water withholding. The trend of decreased photosynthetic rate (Fig. 2.6a) combined with lower stomatal conductance, except for IE 4757 (Fig. 2.6b) and lower transpiration rate (Fig.

2.6c) are similar to those reported for cowpea under similar conditions (Souza, Machado et al. 2004). Significant decrease in P_n of IE 4757, IE 4797, and IE 7079, under water stress can be attributed to increased internal CO_2 concentrations (C_i) and decreased Rubisco activity (Costa França, Pham Thi et al. 2000). After water withholding, significant reduction in E was observed in all the five accessions (Fig. 2.6c). This response can be classified as a strategy of drought avoidance (Anyia and Herzog 2004). The same study presents improved WUE of a few cowpea genotypes under water stress. Increased WUE has also been reported in some varieties of rice, where a significant increase of ~46% was observed under stress (Cabuslay, Ito et al. 2002). Similar to these observations, our results show improved WUE for two accessions: IE 6537 and IE 7079 (Fig. 2.6d).

2.4.3 Chlorophyll and carotenoid contents

The photosynthetic pigment chlorophyll plays a crucial role in photosynthesis. With low availability of water, membranes become damaged and chloroplasts become degraded (Alberte, Thornber et al. 1977). Plants overcome oxidative assault caused during drought stress by either enzymatic or non-enzymatic antioxidant defenses. Higher accumulation of pigments (carotenoids) under stress helps plant protect themselves by getting rid of excessive energy by thermal dissipation (Reddy, Chaitanya et al. 2004).

Under water stress, our study showed a variety of responses in all five accessions for chlorophyll and carotenoid accumulation. Significant reductions of total chlorophyll (TC) were seen in IE 4797 and IE 7079 (Fig. 2.7c) which can be

attributed to increased activity of chlorophyllase enzyme or destruction of chloroplast (Djanaguiraman, Sheeba et al. 2006). A 45% decline in the total chlorophyll content (TC) in water stressed plants of finger millet, which were maintained at 30% field capacity (FC) for 8 days, was previously reported (Parvathi, Nataraja et al. 2012). Our findings are consistent with their report, as IE 4797 shows a significant decline of 28% and IE 7079 shows a significant decline of 39%, when water was withheld for 6 days. On the other hand, significant accumulation of total chlorophyll (TC) was seen in IE 4757, IE 5091 and IE 6537 (Fig. 2.7c), which indicate that these three accessions were able to withstand the water stress and continue their normal activity.

2.5 Conclusion

Having measured 12 physiological parameters for five finger millet accessions previously selected to represent a range of responses to water withholding, our findings indicate that each accession responds to water withholding in a unique way.

Three accessions (IE 4757, IE 4797 and IE 7079) experienced significant declines in photosynthetic rate (P_n) and transpiration rate (E) (Fig. 2.6 a-c). P_n and E are agronomically relevant parameters, since reduced photosynthesis leads to reduced yield. On this basis, these accessions were the most sensitive to water-withholding, with IE 4797 showing the highest reduction in P_n and E, very closely followed by IE 7079. IE 7079 displayed the greatest decrease in RWC (2.2 fold, Fig. 2.2) corresponding to the most visible wilting (Fig. 2.1), a significant

increase in ion leakage (5.1 fold, Fig. 2.5), greatest increase in proline content (1.4 fold, Fig. 2.3), and greatest decrease (1.6 fold, Fig. 2.7c) in total chlorophyll of any variety tested. Thus, we concluded that IE 7079 was the most sensitive accession we tested, followed by IE 4797 which also recorded low RWC, and had decreased photosynthesis and decreased total chlorophyll, with increased membrane damage and proline accumulation, although the magnitude of these decreases was smaller than IE 7079. Although IE 4757 showed significant declines in P_n and E, this line showed no measurable differences in RWC (Fig. 2.2) or ion leakage (Fig. 2.5) and is therefore not considered to be as sensitive to water withholding as IE 7079 or IE 4797.

Two accessions, IE 5091 and IE 6537, showed a decrease in RWC but no significant changes in P_n (Fig. 2.2 and Fig. 2.6a). However, IE 5091 did not exhibit an increase in ion leakage following stress, whereas IE 6537 did. Furthermore, IE 5091 did not show a significant increase in proline accumulation, as was observed in other lines. Because IE 5091 showed the smallest decline in RWC (1.1 fold), no significant ion leakage (Fig. 2.5) and no significant proline accumulation (Fig. 2.3), we concluded that IE 5091 was the most tolerant accession. This accession was unique in its ability to maintain its photosynthetic capacity (Fig. 2.6a) with increased chlorophyll (Fig. 2.7c) and anthocyanin content (Fig. 2.4).

2.6 References

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2.7 Appendix

2.7.1: Preliminary screen for 83 accessions of finger millet. Screenings were conducted by withholding water supply for 3 days and assess plants based on visual scoring. (×: started wilting after 3 days of water stress✓: Tolerant to water stress after 3 days of water stress)

Accession lines	Screening	Accession lines	Screening
IE 2589	✓	**IE 7018**	✓
IE 3077	×	**IE 4326**	✓
IE 4622	✓	**IE 2042**	×
IE 7079	×	**IE 4073**	×
IE 2217	×	**IE 2437**	×
IE 5106	×	**IE 3317**	✓
IE 4545	×	**IE 3392**	✓
IE 4057	✓	**IE 2296**	×
IE 6421	✓	**IE 2619**	✓
IE 6473	×	**IE 3391**	✓
IE 6537	×	**IE 2790**	✓
IE 6514	✓	**IE 2430**	✓
IE 3952	×	**IE 1055**	×
IE 4570	✓	**IE 5091**	×
IE 5817	×	**IE 4797**	✓
IE 4646	×	**IE 4795**	✓
IE 5367	×	**IE 4491**	×

IE 2872	×	**IE 4757**	×
IE 2871	✓	**IE 7320**	✓
IE 2606	×	**IE 2821**	✓
IE 2572	✓	**IE 3614**	✓
IE 3104	✓	**IE 4734**	✓
IE 5537	✓	**IE 6240**	✓
IE 6059	✓	**IE 5201**	✓
IE 3045	✓	**IE 3945**	✓
IE 2957	✓	**IE 4121**	✓
IE 2911	✓	**IE 4565**	✓
IE 2457	✓	**IE 5066**	✓
IE 3475	×	**IE 6326**	✓
IE 6165	✓	**IE 4673**	✓
IE 6154	✓	**IE 3618**	✓
IE 6221	✓	**IE 2403**	✓
IE 6082	✓	**IE 5870**	✓
IE 3721	✓	**IE 4816**	✓
IE 6082	✓	**IE 4497**	✓
IE 3721	✓	**IE 4028**	✓
IE 6350	✓	**IE 5306**	✓
IE 6337	✓	**IE 2312**	✓
IE 4671	✓	**IE 6294**	✓
IE 518	✓	**IE 3973**	✓

IE 501	✓	**IE 2710**	✓
IE 2034	✓	**IE 4709**	✓
IE 3470	✓		

Chapter 3 – Marker discovery and population studies in finger millet (*E. coracana* (L.) Gaertn.)

3.1 Introduction

Finger millet (*Eleusine coracana* (L.) Gaertn.) is a self-pollinating allotetraploid (AABB, 2n=4x=36) that has been cultivated as a cereal crop since its domestication in Africa 5000 years ago (Dida, Ramakrishnan et al. 2007). Domesticated finger millet is sometimes designated as *E. coracana* subsp. *coracana* to distinguish it from its wild progenitor, *E. coracana* subsp. *africana*. The donor of the A genome is presumed to be *E. indica*. For the B genome, *E. floccifolia* had been proposed as the best candidate donor species. However, evidence from nuclear and plastid sequences showed that *E. floccifolia* was not the B genome donor (Neves, Swire-Clark et al. 2005; Liu, Triplett et al. 2011). Another congener, *E. tristachya*, had also been proposed as a possible B genome donor, but subsequent genomic *in situ* hybridization experiments indicated that *E. tristachya* was more closely related to *E. indica* than to *E. floccifolia* (Bisht and Mukai 2002). The B genome donor of finger millet may therefore be extinct.

Finger millet is a member of the Chloridoideae subfamily of grasses (Poaceae), and so is more closely related to tef (*Eragrostis tef*) and bermuda grass (*Cynodon dactylon*) than to other millets or more familiar cereals. The finger millet genome has been measured at 1.8 pg/1C nucleus by flow cytometry (Dida, Ramakrishnan et al. 2007), corresponding to approximately 2.5 Gbp (Bennett and Leitch 1995), which is much larger than other sequenced cereal genomes, such as

that of rice (430 Mbp) (Pennisi 2007), but much smaller than hexaploid wheat (*Triticum aestivum*, 17 Gbp (Brenchley, Spannagl et al. 2012)).

Table 3.1: Molecular marker sets identified in *Eleusine coracana*.

Row	Marker types	Accessions assessed	References
1	11 RFLP, 18 RAPD, 6 ISSR	22 accessions of 5 species of *Eleusine*	(Salimath, Oliveira et al. 1995)
2	50 RAPD	32 accessions	(Babu, Senthil et al. 2007)
3	18 RAPD, 10 SSR, 10 cyt P_{450}	83 accessions	(Panwar, Saini et al. 2010)
4	25 RAPD	15 accessions	(Das and Misra 2010)
5	21 RAPD, 24 SSR	52 accessions	(Kumar, Sharma et al. 2012)
6	17 SSR	67 accessions	(Arya, Verma et al. 2013)

Molecular markers are valuable in population studies, phylogenetics, varietal identification, gene mapping, and in breeding. Isozymes were the first markers used in finger millet to demonstrate its allotetraploid origin (Werth, Hilu

et al. 1994). Isozyme markers have also been used to distinguish the *africana* and *coracana* subspecies and also to demonstrate ancestral relationship to *E. indica*. Existing genetic marker resources for finger millet are outlined in Table 3.1. A research group (Salimath, Oliveira et al. 1995) aimed to understand the genetic diversity amongst five species of *Eleusine* using three marker systems: RAPD, RFLP and inter simple sequence repeats (ISSR). They found considerable polymorphism between the species and concluded that ISSRs were the most effective in defining genetic diversity. RAPD markers have also been used to select diverse germplasm for breeding (Babu, Senthil et al. 2007). Markers associated with calcium content (Panwar, Nath et al. 2010) and protein content (Kumar, Sharma et al. 2012) have also been identified.

Several reports of SSR markers in finger millet have been published to date. SSRs have advantages over other types of markers (e.g. RFLP, RAPD), in that they are usually co-dominant, often multi-allelic and do not require radioactivity for detection (Henry 2001). The first set of SSRs in finger millet was developed as a part of a linkage mapping study of a F_2 population derived from a cross between subsp. *coracana* and *africana* (Dida, Ramakrishnan et al. 2007). Of the 42 SSRs identified, 31 polymorphic markers were mapped. Transferability of 17 SSRs to pearl millet was studied (Arya, Verma et al. 2009). A new set of 10 SSRs (Panwar, Nath et al. 2010) and 24 SSR markers (Kumar, Sharma et al. 2012) were used to study the genetic diversity of the 52 accessions of finger millet collected from Uttarakhand, India. Later, three genic makers (Arya, Verma et al. 2013) and 14 previously identified SSRs (Dida, Ramakrishnan et al. 2007), were

used to analyze 67 diverse accessions of finger millet native to India and Africa. Arya and colleagues reported that the 17 SSRs used to assess genetic diversity in 67 accessions of finger millet yielded a total of 69 alleles with a mean of 4 alleles per locus and a gene diversity of 0.471. They also grouped these accessions into structured sub-populations based on diversity assessed using SSR markers and suggested that these populations could be used for finger millet germplasm enhancement (Arya, Verma et al. 2013).

The objective of our study is to increase the available DNA sequence information and expand genetic marker resources for finger millet, and to use this information to characterize the diversity of existing germplasm collections. In the future, these markers may also be used in mapping and marker assisted selection to enhance finger millet breeding.

3.2 Materials and Methods

3.2.1 Plant material

A total of 83 accessions of *E. coracana* obtained from ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India) were used in the present study. These accessions were selected from a mini-core collection (Upadhyaya, Sarma et al. 2010) to represent diverse geographic origins (Appendix 3.1). In addition, eight wild relatives of *E. coracana* obtained from U.S. Department of Agriculture (USDA, Appendix 3.2) were also used in this study.

Seeds were sown in Sunshine Mix 4 (Sun Gro Horticulture, USA) ~200 g/pot, planted in round pots (9 cm height, 10 cm diameter at the top), to a depth of approximately 1 cm, at a density of 7-8 seeds per pot. Plants were grown in environmental growth chambers at 29 °C with ~42% relative humidity, and a photosynthetic photon flux (PPF) of 110 ± 10 μmole m^{-2} s^{-1} supplied by high output fluorescent bulbs on a 12 h light/12 h dark cycle. Eight day old seedlings were used for DNA extraction.

For RNA Seq experiments, initial plant growth conditions were the same as described above. After germination, seedlings of the six selected accessions (Table 3.2) were thinned to one seed per pot. All plant were regularly watered for up to 26 days after seeding (DAS). At 14 DAS plants were fertilized with Plant-Prod 20-20-20 water soluble fertilizer (Plant Prod, Ontario, Canada). For plants in the treatment group, water withholding was initiated 26 DAS, while the control group was grown in parallel and was watered until the soil was saturated. Tissues were harvested 36 hours (i.e. 28 DAS) after the imposition of stress. Meristematic tissues from the bottom part of the stem along with youngest leaves were collected for three lines (Table 3.2) and immediately flash froze in liquid nitrogen. The same was done for floral tissue and root tissue. These samples were then processed for RNA extraction.

Table 3.2: Accessions of finger millet received from ICRISAT and used for RNA sequencing. All tissues were collected at 28 DAS. Water stressed plants had water withheld for 36 hours.

Sample No.	Accession No. (ICRISAT)	No. of biological replicates sequenced	Geographic origin	Tissue description	Treatment	RNA integrity number
1	IE 4709	1	Burundi	Flower	Well watered	8.31
2	Anonymous 1	1	Unknown	Leaf	Well watered	8.20
3	Anonymous 2	1	Unknown	Root	Water stressed	8.36
4	Anonymous 3	1	Unknown	Root	Well watered	7.95
5	IE 7079	2	Kenya	Leaf	Well watered	8.30; 8.30
6	IE 7079	2	Kenya	Leaf	Water stressed	7.70; 7.10
7	IE 6537	2	Nigeria	Leaf	Well watered	7.20; 8.20
8	IE 6537	2	Nigeria	Leaf	Water stressed	8.50; 7.80

3.2.2 RNA extraction and sequencing

Total RNA was extracted from frozen young leaf blades of 28 DAS finger millet (both control and treatment). Extractions were done with a combined CTAB – Qiagen protocol as previously described (Johnson, Carpenter et al. 2012). Extracted RNA was first quantified using a spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies LLC, Wilmington, Delaware, USA). RNA samples with a concentration of 150-300 ng μl^{-1} were tested for quality on a 2100 Bioanalyzer (Agilent Technologies Inc., Germany). Samples with a RNA integrity number (RIN) greater than 7.0 were selected and were sequenced on an Illumina Genome Analyzer 2 (Beijing Genomics Institute). Raw reads for ICRISAT accessions IE 6537 and IE 7079 were deposited in the NCBI SRA database as BioProject 2420128. Raw reads and assemblies for other samples were deposited in the One Thousand Plants (1KP) project database at www.onekp.com. Raw reads from samples 1 through 4 (Table 3.2) were combined and assembled using SOAPdenovo as described previously (Johnson, Carpenter et al. 2012). SSR regions were identified within the transcripts using the Misa software package (Thiel, Michalek et al. 2003), with definition settings 1-100 2-9 3-6 4-5 5-6 6-5. Primer3 was used to design primers to surround these regions (Rozen and Skaletsky 1998).

3.2.3 DNA extraction

8 day old young leaves (~30 mg) were placed in 2 ml eppendorf tubes and immediately flash frozen. These tubes were then processed in a MixerMill

(Retsch MM30, Germany). A CTAB extraction protocol, previously described (Mace, Buhariwalla et al. 2003) was followed for DNA extraction, followed by quantification using a spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies LLC, Wilmington, Delaware, USA).

3.2.4 SSR marker analyses

For initial screening of SSR primer pairs, 50 μL PCR reactions were made using 1.0 μL genomic DNA (~50-100 ng), 5 μL of 10X PCR buffer (200mM Tris-Cl pH8.3, 500 mM KCl, 15 mM $MgCl_2$), 1.0 μL of 10 mM dNTP, 0.5 μL of Taq DNA polymerase, 1 μL reverse primer and 1 μL of the forward primer. PCR conditions were: 95°C (5 min), followed by 39 cycles at 94°C (1 min), 60°C (1 min), 72°C (2 min) and a final extension at 72°C (2 min). These PCR products were separated on a 4% super fine agarose gels (Metaphor Agarose, Cambrex Bio Science, UK), using the Orange G ladder (Sigma-Aldrich, Missouri, USA).

For the 95 selected SSR markers, primers were re-synthesized with an M13 (-21) tail on their 5' ends and used for genotyping as previously described (Schuelke 2000). Each of the tagged markers was labeled with FAM, VIC, NED, or PET (Applied Biosystems). PCR amplifications consisted of 25 μL of reaction mixture. Each reaction was made up of 0.5 μL genomic DNA (~50-100 ng), 2.5 μL of 10X PCR buffer (200mM Tris-Cl pH8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.5 μL of 10 mM dNTP, 0.25 μL of Taq DNA polymerase, 8 pmol of M-13 tailed reverse primer and 2 pmol of the forward primer. Cycling conditions were: 94°C (5 min), followed by 39 cycles at 94°C (45 s), 50°C (30 s), 72°C (45 s) and a final extension at 72°C (7 min). 2 μL of 10-fold diluted PCR product was added to 8

μL of Hi-Di and 0.2 μL of 500 LIZ Size Standard (Applied Biosystems) and run on an ABI Prism Genetic Analyzer (Applied Biosystems). DNA sizing and allele calling were conducted using GeneMapper software (GeneMapper v3.0, Applied Biosystems).

3.2.5 Allelic diversity analysis

31 polymorphic SSR primer pairs, including novel primers described here as well as previously published primers (Dida, Ramakrishnan et al. 2007; Kumar, Sharma et al. 2012), were used to genotype 83 different accessions obtained from ICRISAT. 81 of the original 95 primer pairs were used to assess the relationship among the wild accessions of *Eleusine*, which were obtained from USDA Plant Genetic Resources Conservation Unit, Griffin, Georgia, USA (Table 3.3). Allelic frequencies, polymorphism information content (PIC), and other descriptive statistics were calculated using Polysat (Clark and Jasieniuk 2011) and PowerMarker V3.0 (Liu and Muse 2005).

Table 3.3: Wild relatives of domesticated *E. coracana* analyzed in this study.

S. No.	Species	USDA accession number
1.	*Eleusine coracana* subsp. *africana*	PI 226270
2.	*Eleusine coracana* subsp. *africana*	PI 315700
3.	*Eleusine floccifolia*	PI 196853
4.	*Eleusine indica*	PI 217609
5.	*Eleusine indica*	PI 408803
6.	*Eleusine indica*	PI 442480

7.	*Eleusine multiflora*	PI 230637
8.	*Eleusine tristachya*	PI 442481

3.2.6 Clustering analysis

For construction of the dendrogram, SSR genotypes were coded in a binary table of alleles using Polysat (Clark and Jasieniuk 2011). This table was imported into NTSYSpc (Exeter Biological Software; (Rohlf 1993)), where the SIMQUAL module was used to generate a similarity matrix, which was used as input to the SAHN clustering module with default settings.

3.3 Results

3.3.1 Transcriptome sequencing and assembly

We sequenced transcriptomes of *E. coracana* to increase the available information about its genes, especially those related to stress responses, and to provide a resource for the identification of additional SSR loci. In the first set of transcriptome sequencing experiments, RNA was obtained from four accessions that had been subjected to water withholding (Table 3.2). The RNA was extracted from leaves, roots and flowers to represent a broad diversity of expressed genes. Following further analysis of physiological responses to water withholding (Chapter 2) two other accessions (IE 7079 and IE 6537) were selected for quantitative analysis of gene expression using RNA sequencing (Table 3.2).

These data are being analyzed by other lab personnel to detect differential expression between stressed and unstressed plants.

3.3.2 SSR locus identification

From the combined transcript assemblies of four RNA samples (Samples 1-4, Table 3.2), 3376 putative microsatellites were identified for which SSR primers could be synthesized. Of these 3376 primer pairs, 288 were prioritized based on several criteria including a preference for amplicons with the highest proportion of SSR length compared to total amplicon length. These 288 primer pairs were synthesized and screened empirically as follows.

3.3.3 SSR screening

The 288 novel primer pairs developed at the University of Alberta were initially screened using high-resolution agarose gel electrophoresis of their PCR products. Following optimization of PCR conditions with a subset of these primers (Fig. 3.1), all 288 primer pairs were used to amplify genomic DNA from 24 randomly selected accessions from the ICRISAT mini-core collection (Upadhyaya, Sarma et al. 2010) (Fig. 3.2). Based on these results, 47 primer pairs were selected that gave good fragment resolution and product amplification.

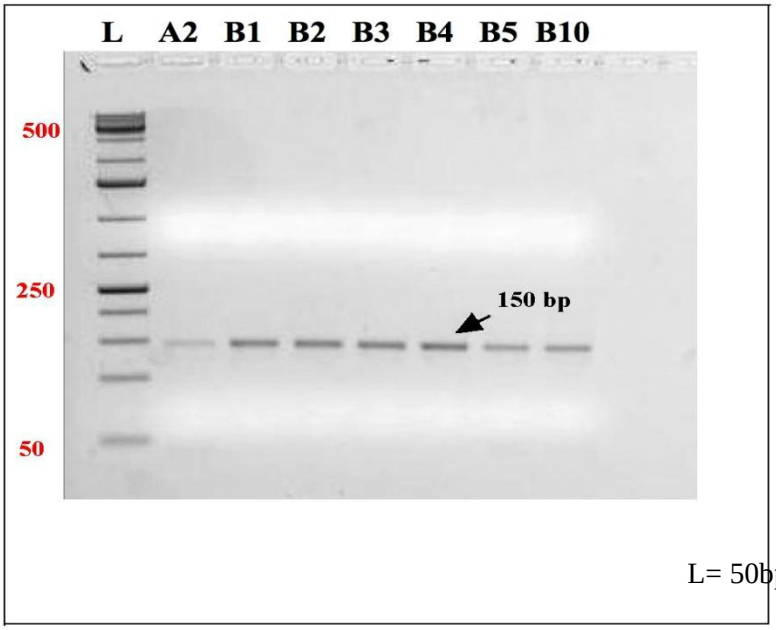

Figure 3.1: Gel run for primer ECS 1003 with 6 different genotypes (A2: IE 3077, B1: IE 7079, B2: IE 2217, B3: IE 5106, B4: IE 4545, B5: IE 4057, B10: IE 3952).

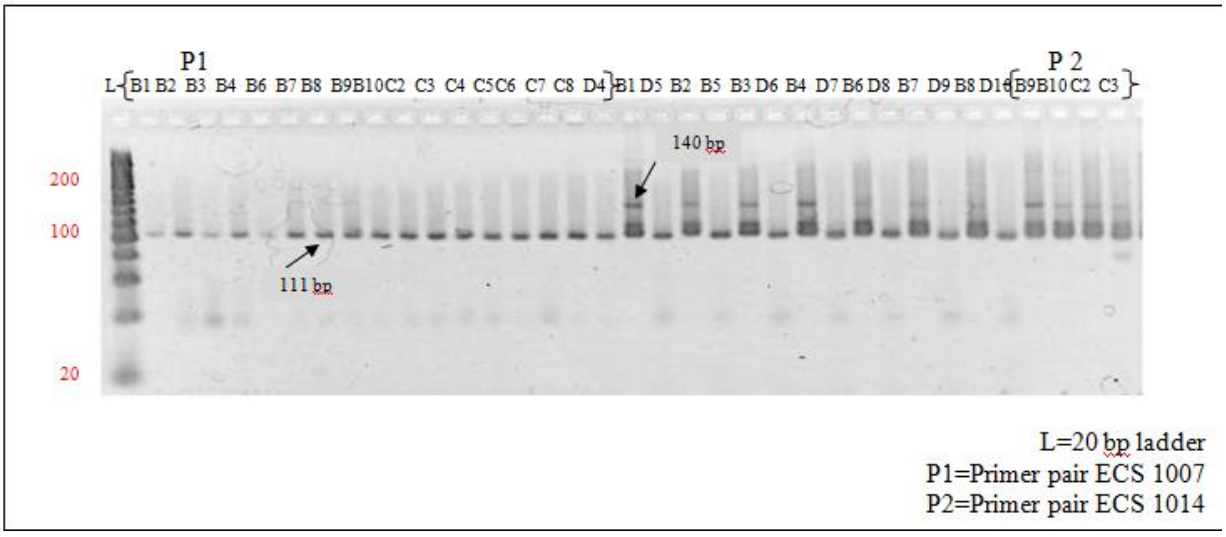

Figure 3.2: Gel run showing product amplification of 24 different DNA templates when amplified with ECS 1007 and ECS 1014.

These 47 novel primer pairs, as well as 48 previously published primer pairs (Dida, Ramakrishnan et al. 2007; Kumar, Sharma et al. 2012), were then re-synthesized with an M-13 tail to facilitate fluorescent labeling for analysis using an ABI Prism Genetic analyzer (Schuelke 2000). 83 accessions of finger millet from the ICRISAT mini-core collection (Upadhyaya, Sarma et al. 2010) were analyzed using these 95 primer pairs.

The first round of analysis using the 95 primer pairs resulted in the selection of 76 primer pairs that successfully amplified genomic DNA from 24 accessions (randomly selected from the ICRISAT mini-core collection). These included 43 novel primers and 33 primers that were previously published (Dida, Ramakrishnan et al. 2007; Kumar, Sharma et al. 2012). Of the 76 primer pairs, 43 were found to amplify polymorphic products, and these 43 pairs were then used to genotype the remaining 59 accessions from the ICRISAT mini-core collection. Ultimately, 31 primer pairs (Table 3.4) were chosen that reliably produced interpretable peak patterns in almost all the accessions from the ICRISAT collection. Of these, 23 were novel and eight were previously published. The remaining primer pairs were deemed un-scorable based on unexpectedly high peak number (Fig. 3.3) or inconsistent peak height (Fig. 3.4).

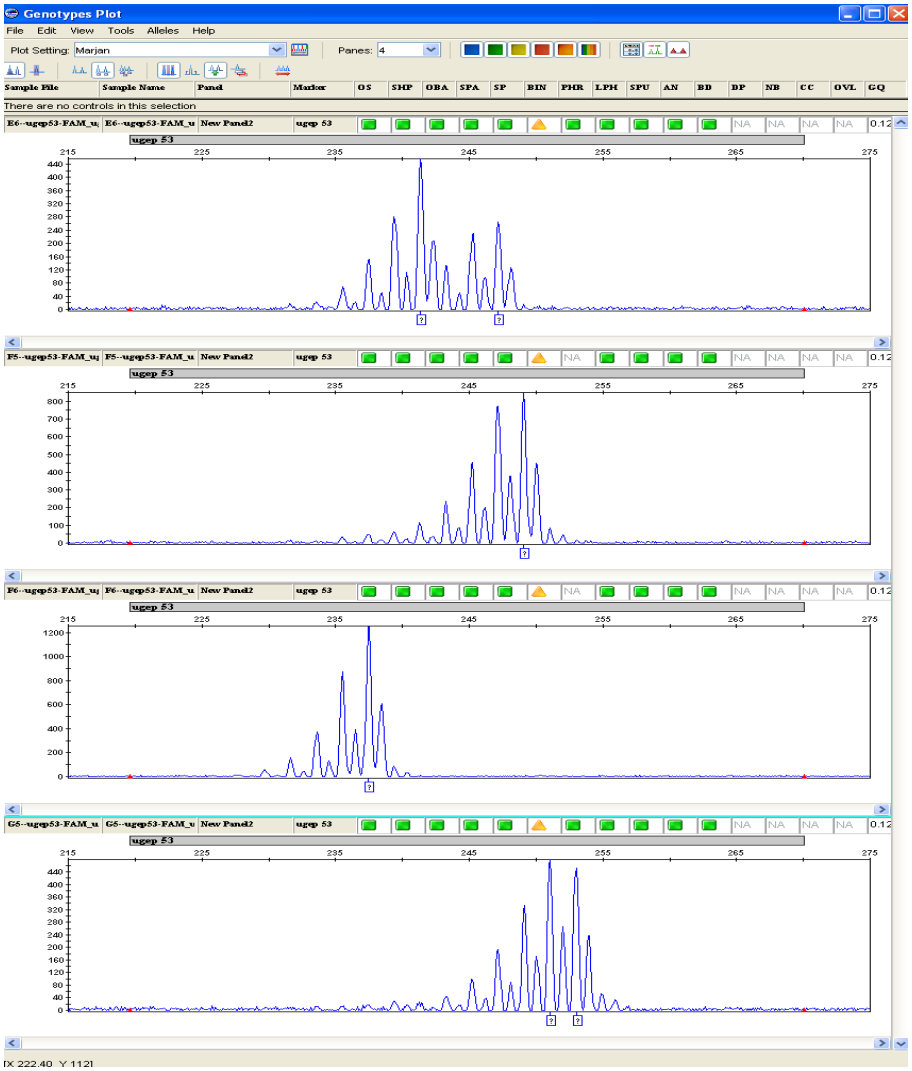

Figure 3.3: Output of ABI prism showing high peak number. Analysis using GeneMapper V4.0. SSR marker UGEP 53 showed unexpected peak morphology on amplifying DNA of IE 7320, IE 2457, IE 3475 and IE 518.

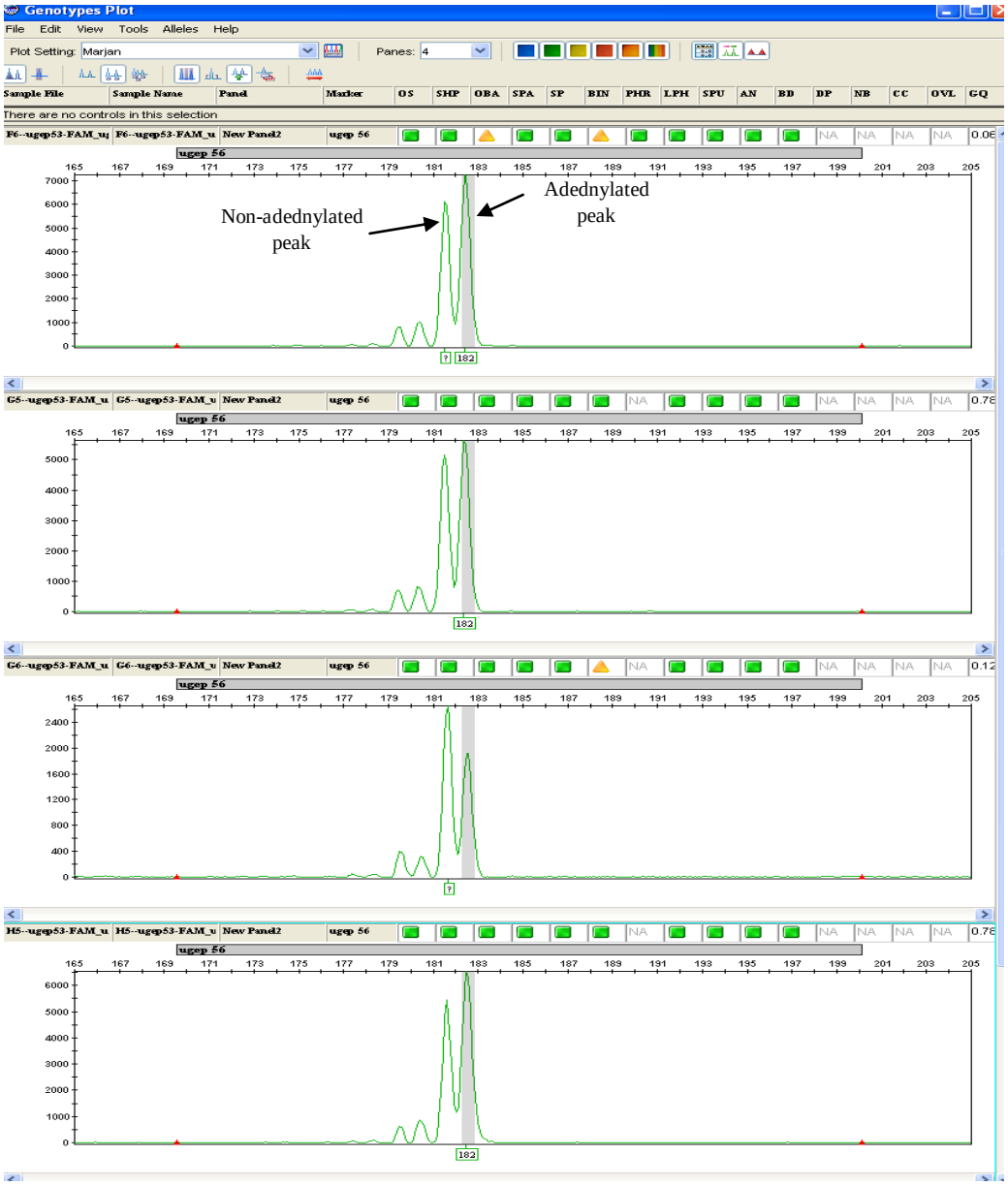

Figure 3.4: Peak height ratios. SSR marker UGEP 56 showing unexpected peak height ratios on amplifying DNA of IE 3475, IE 518, IE 3614 and IE 4673.

Table 3.4: Characteristics of 31 SSR markers used to study polymorphism in 83 accessions of finger millet. Details of primer sequence, repeat length and expected product size.

S. No.	SSR markers	Primer sequence	SSR motif	T_m value of primer(C°)	Amplicon size (bp)
1.	ECS 201R	CAC AAC ACC CAG CAT AGC AT	$(ATGCT)_5$	55.64	109
	ECS 201F	TAG ATC CGT CAA GGA CCA CC			
2.	ECS 204R	CCC AAG CAC ATC ATG TAA GC	$(TGAT)_5$	54.15	105
	ECS 204F	GTC GTC CAC CAT GAA GCT G			
3.	ECS 207R	TAT CTC CGC CAT CCA ATA GC	$(AGC)_6$	54.2	256
	ECS207F	CCA CCA GAC AGA CGA GGA AT			
4.	ECS 208R	ACC ATA ATA GGG CCG CTT G	$(GT)_{11}$	54.92	162
	ECS 208F	CTC CGA TAC AGG CGT AAA GG			
5.	ECS 209R	CTT GAT AAT GAA TGG AGG CAC A	$(TC)_9$	52.94	99
	ECS 209F	GTA TCA GAT TTT CCT GGC CC			

6.	ECS 211R	TCG CAA AAC TCA CAG ACA GC	$(CGC)_6$	55.81	100
	ECS 211F	CTT GGG AGG AGA ACA ACC TG			
7.	ECS 213R	CTC CAC ACC GAC CAG CTT A	$(TGTCG)_5$	56.79	140
	ECS 213F	CCT ATC AAC AAA AGC ACG CA			
8.	ECS 216R	CAA GCG TGA TTT TCC TGT GA	$(AATCT)_5$	53.38	115
	ECS 216F	TCT GGC TCA TTT CCT GTC CT			
9.	ECS 218R	TGC CCT GCT GTC GAT CAA T	$(TC)_{10}$	56.93	99
	ECS 218F	TCT CTC TCT CTC TCT CTC TCT ACG TC			
10.	ECS 219R	CGG ATC ACC ACG TCA TTA AA	$(ATC)_7$	52.65	104
	ECS 219F	AGC ACC TTC ACA TGC ACC			
11.	ECS 220R	ACA ACA AAC TGC ACT GCC TG	$(AGAC)_5$	56.43	101
	ECS 220F	CTT GGT TGG TTG GTT GCT TT			
12.	ECS 222R	GTG CCC GAA TCG AGT AAA AT	$(TC)_{10}$	52.99	103
	ECS 222F	TCT CTC TCT CTC TCT CTC TCA GAC			

		TAC			
13.	ECS 226R	CAA TGT TGC CCA GGA CCT AT	$(GA)_{10}$	54.94	107
	ECS 226F	TTC GGT AGC CAT TTC TCT GTC			
14.	ECS 227R	CTC AAC GAA TTC CTT CCA GC	$(TTA)_7$	53.76	114
	ECS 227F	TTT TGG GGT GGA GTT TCT TG			
15.	ECS 228R	TCT CCC TCA ACG TCT CTC GT	$(GGA)_6$	57.34	255
	ECS 228F	GCA GGT CGT CTC TGA ACC TC			
16.	ECS 232R	ACT CCA CCG AAA GTC ACC AC	$(CGA)_6$	57.18	275
	ECS 232F	GCG TCC CAT CTC TTC TTC AG			
17.	ECS 233R	TGC TGT GCT CCT CTG TTG AC		57.45	175
	ECS 233F	AAT TCC ATT CTC TCG CAT CG			
18.	ECS 234R	TCA TTG ATG AAT CCG ACG TG		52.69	232
	ECS 234F	CGG TCA GCA TAT AAC GAA TGG			
19.	ECS 237R	GGT GGT TGC ACA CGT AGA GG	$(CAA)_6$	58.24	99

	ECS 237F	ATC GCC GGT CCT TTT TCA T			
20.	ECS 241R	TGC ATG TTT CTG TTA AAT GCC	$(GAT)_6$	52	101
	ECS 241F	GCA ATG TCT ACC AGA TGA TGA TG			
21.	ECS 244R	ATT GCC ATT GGT GGT GGT	$(CCA)_6$	55.05	103
	ECS 244F	CAT CAT CAA ATC ATG CAG CAG			
22.	ECS 245R	CCG GAG ATT CAT CTT CCT CC	$(CAA)_6$	54.64	105
	ECS 245F	ACT TCC TCG CCG AAA TCC			
23.	ECS 246R	ATG CGA CGA AGG GAG TGA T	$(CGC)_6$	56.3	104
	ECS 246F	AAA CAA CCC TAA GCT CAC CG			
24.	Ugep106R	TGC TGT GCT CCT CTG TTG AC	$(AC)_{12}$	65.5	175
	Ugep 106F	AAT TCC ATT CTC TCG CAT CG			
25.	ugep 68R	TCA TTG ATG AAT CCG ACG TG	$(CT)_{14}$	66.35	232
	ugep 68F	CGG TCA GCA TAT AAC GAA TGG			
26.	ugep 11R	AAG ACG CTG GTG GAA ATA GC	$(CT)_{12}$	69.08	153

	ugep 11F	CCT CGA GTG GGG ATC CAG			
27.	ugep 12R	TCA AAG TGA TGC GTC AGG TC	$(CT)_{22}$	67.59	230
	ugep 12F	ATC CCC ACC TAC GAG ATG C			
28.	ugep 26R	TGT CCC TCA CTC GTC TCC TC	$(CGG)_7$	67.3	227
	ugep 26F	ATG GGG TTA GGG TTC GAG TC			
29.	ugep 31R	CCG TGA GCC TCG AGT TTT AG	$(GA)_{12}$	65.68	241
	ugep 31F	ATG TTG ATA GCC GGA AAT GG			
30.	ugep 52R	TGC TGG GTG AAA CCC TAG AC	$(GA)_{16}$	66.74	215
	ugep 52F	TCA TGC TAG CTT CAA CAC AAC C			
31.	fmssr 12R	CGT CTT CTC ATG CAT GGG GGC GC		69.9	188
	fmssr 12F	CGA TCC ATT CCT GCT GCT CGC			

3.3.4 SSR genotyping of diverse accessions of cultivated *E. coracana*

Analysis of the genotyping results obtained from ABI Prism analysis of 83 ICRISAT accessions with the 31 scorable primer pairs showed that a total of 152 alleles were identified and polymorphism was found to range from 2 to 13 alleles per locus, with an average of 4.9 alleles per locus (Table 3.6). Two of the primer pairs (ECS 211 and ECS 246) amplified only two alleles. Polymorphism information content (PIC) for the 31 scorable primer pairs ranged from 0.024 to 0.853 with a mean of 0.495.

Table 3.5: Summary of SSR markers used to evaluate genetic diversity of finger millet.

	ICRISAT accessions	**USDA accessions**
No. of accessions	83	8
No. of SSRs scored	31	81
Total no. of alleles	152	331
Mean PIC	0.495	0.531

Table 3.6: Allelic diversity attributes of 31 SSR markers. Markers were genotyped on 83 accessions of finger millet obtained from ICRISAT. Sample size (n=32), PIC: polymorphism information content

Marker	Major Allele Frequency	Allele No.	PIC
ECS 201	0.988	3	0.024
ECS 204	0.506	4	0.512
ECS 207	0.955	4	0.088
ECS 208	0.599	5	0.508
ECS 209	0.36	7	0.713
ECS 211	0.506	2	0.5
ECS 213	0.616	5	0.548
ECS 216	0.543	4	0.518
ECS 218	0.685	3	0.448
ECS 219	0.835	5	0.292
ECS 220	0.504	3	0.528
ECS 222	0.9	6	0.185
ECS 226	0.683	5	0.451
ECS 227	0.756	3	0.407
ECS 228	0.681	3	0.466
ECS 232	0.786	5	0.337
ECS 233	0.596	5	0.552
ECS 234	0.71	5	0.463
ECS 237	0.94	3	0.116

ECS 241	0.494	4	0.583
ECS 244	0.527	6	0.522
ECS 245	0.294	5	0.799
ECS 246	0.506	2	0.5
fmssr12	0.301	7	0.776
ugep106	0.553	7	0.593
ugep11	0.492	5	0.605
ugep12	0.23	13	0.853
ugep26	0.333	5	0.724
ugep31	0.679	6	0.506
ugep52	0.401	7	0.736
ugep68	0.713	5	0.46

A dendrogram (Fig 3.5) was obtained from the binary data deduced from the DNA profiles of the 83 ICRISAT finger millet accessions. Genotypes that were genetically similar (based on allele sharing) were clustered together. Three major clusters were observed. Cluster I consisted of the accession IE 4709 form Burundi; which was otherwise distinct among the ICRISAT mini-core collection because of its relatively small seeds and because it was the only ICRISAT accession to flower in our growth chamber. The remainder of accessions in the dendrogram could be divided into two large clusters (cluster II and cluster III). Both clusters were comprised of varieties with broad geographic origins. Cluster

II had two sub-clusters within it (cluster II-a and cluster II-b), where one of them strictly comprised of accessions from Asia (India and Nepal), whereas the other group was a combination of accessions from all over the world. Cluster III was also comprised of two sub-clusters (cluster III-a and cluster III-b). One group was strictly made up of accessions from Uganda whereas the other cluster was a combination of varieties from all over the world.

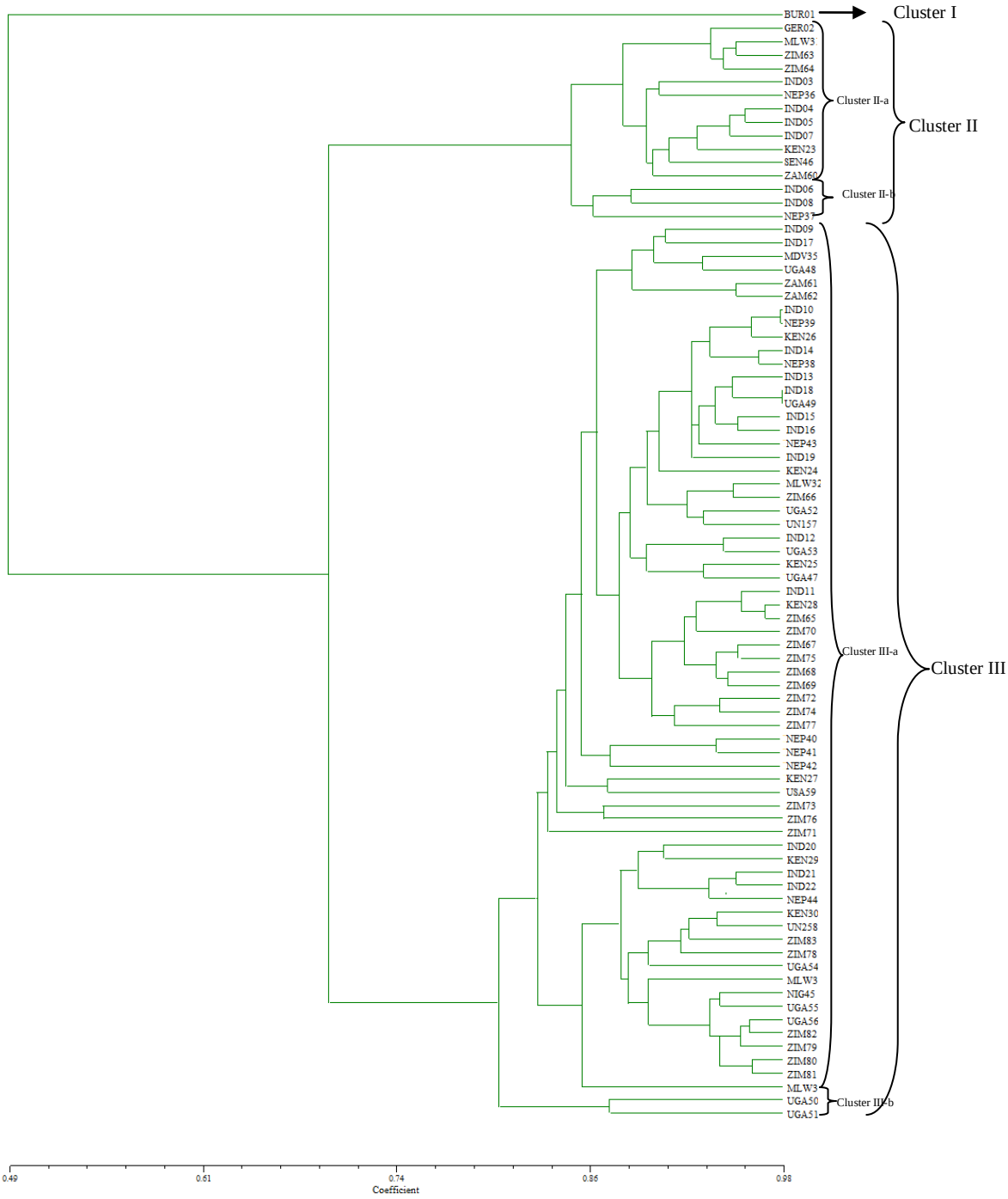

Figure 3.5: Dendrogram for the 31 SSR markers data of 83 ICRISAT accessions. Cluster I comprises the flowering accession from Burundi, cluster II and cluster III comprise varieties from various geographic regions. An index to the abbreviated names shown in this dendrogram is found in Appendix 3.7.1

3.3.5 SSR genotyping of wild relatives of cultivated *E. coracana*

To further understand the relationships between *E. coracana* subspecies and their congeners, and to test the transferability of our markers to other species, we tested the 95 primer pairs (47 novel markers and 48 published markers) on the wild relatives of *E. coracana* that were obtained from USDA (Table 3.3).

Of the 95 primer pairs tested on the eight different accessions, 81 were found to be scorable. This included 47 novel primer pairs and 34 published primer pairs. A total of 331 alleles were generated and polymorphism was found to range from 1 to 9 alleles, with an average of 4 alleles per locus (Table 3.7). 15 primer pairs amplified two or fewer bands. Polymorphism information content (PIC) for the 81 scorable primer pairs averaged to 0.531.

Table 3.7: Allelic diversity attributes of SSR markers used to genotype DNA obtained from five relatives of *E. coracana*. Sample size (n=81), PIC: polymorphism information content

Marker	Major Allele Frequency	Allele No.	PIC
ECS200	0.625	3	0.428
ECS201	0.313	6	0.748
ECS202	0.438	5	0.690
ECS203	0.625	4	0.510
ECS204	0.500	6	0.666
ECS205	0.500	3	0.511
ECS206	0.625	5	0.539

ECS207	0.313	5	0.737
ECS208	0.375	6	0.754
ECS209	0.750	3	0.371
ECS210	0.625	4	0.524
ECS211	0.750	3	0.371
ECS212	0.625	4	0.524
ECS213	0.438	5	0.668
ECS214	0.625	3	0.468
ECS215	0.250	9	0.835
ECS216	0.375	5	0.722
ECS217	0.188	7	0.833
ECS218	0.375	4	0.667
ECS219	0.375	4	0.667
ECS220	0.500	4	0.605
ECS221	0.250	6	0.757
ECS222	0.375	5	0.682
ECS223	0.625	3	0.468
ECS224	1.000	1	0.000
ECS225	0.500	7	0.678
ECS226	0.375	5	0.712
ECS227	0.500	4	0.605
ECS228	1.000	1	0.000
ECS229	1.000	1	0.000

ECS230	1.000	1	0.000
ECS231	0.500	2	0.375
ECS232	0.750	4	0.387
ECS233	0.500	3	0.511
ECS234	0.375	7	0.765
ECS235	0.250	6	0.786
ECS236	0.438	7	0.725
ECS237	0.375	5	0.642
ECS238	0.438	5	0.657
ECS239	0.688	2	0.337
ECS240	0.750	2	0.305
ECS241	0.625	3	0.468
ECS242	0.375	4	0.630
ECS243	0.375	8	0.766
ECS244	0.625	4	0.524
ECS245	0.500	3	0.544
ECS246	0.500	3	0.555
ugep1	0.500	3	0.511
ugep3	0.563	5	0.599
ugep5	0.375	6	0.655
ugep6	0.500	3	0.511
ugep8	0.750	5	0.404
ugep10	0.875	2	0.195

ugep11	0.500	5	0.653
ugep12	0.625	3	0.468
ugep15	0.188	7	0.824
ugep18	1.000	1	0.000
ugep21	0.625	5	0.525
ugep24	0.875	2	0.195
ugep26	0.500	5	0.618
ugep31	0.375	5	0.712
ugep52	0.250	6	0.776
ugep53	0.750	3	0.371
ugep56	0.875	2	0.195
ugep60	0.500	5	0.653
ugep65	0.375	5	0.712
ugep68	0.375	5	0.712
ugep76	0.625	3	0.468
ugep77	0.375	5	0.712
ugep78	0.375	6	0.754
ugep81	0.438	6	0.702
ugep90	1.000	1	0.000
ugep102	0.375	4	0.636
ugep104	0.375	4	0.667
ugep106	0.438	4	0.645
ugep107	0.875	2	0.195

ugep108	0.500	3	0.511
ugep110	0.750	2	0.305
utr4	0.625	3	0.468
utr5	0.500	3	0.555
fmssr12	0.625	2	0.359
Mean	0.545	4	0.531

3.4 Discussion

3.4.1 SSR markers studied on diverse *E. coracana* accessions

Of the 288 novel SSR primer pairs originally designed, 47 pairs were deemed suitable for use in genotyping with the ABI Prism Genetic Analyzer. Of these, 47 novel primers, 24 were polymorphic (Table 3.6). Thus, 51% of the novel primer pairs tested were polymorphic within the 83 accessions of *E. coracana* obtained from ICRISAT. This frequency is comparable to another study that reported 62% variation (Dida, Ramakrishnan et al. 2007).

However, among the 48 previously published SSR markers (Dida, Ramakrishnan et al. 2007; Kumar, Sharma et al. 2012) we tested, only 9 were polymorphic (19%) among the 83 accessions of *E. coracana* (Table. 3.6). This is much lower than in a previous report (Dida, Ramakrishnan et al. 2007) where the same markers were polymorphic (39%) in 151 F_2 progeny generated from a cross between subsp. *coracana* and subsp. *africana*. This variation can be attributed to the genetic makeup of finger millet. Dida's study comprised of progeny from a

cross between finger millets originating from Nepal and Kenya (Dida, Ramakrishnan et al. 2007). On the other hand, the present study comprises of accessions from all over the world.

We were unable to reliably score 23 novel markers and 40 published markers because of inconsistent or complex peak patterns (Fig. 3.3 and 3.4). This may be caused by inconsistent and incomplete addition of adenosine to the microsatellite sequence (Fig. 3.4) (Henry 2001) and appearance of stutter peaks (Fig. 3.5). Stutter is often the output of slippage by the DNA polymerase during PCR. Stutter peaks mark themselves as a series of amplification products smaller than the true peak.

31 SSR primer pairs (Table 3.6) were used for the molecular characterization of 83 ICRISAT accessions of finger millet. An average of 4.9 alleles per locus was generated in this study which is comparable to a mean allele number of 4.0 previously reported (Arya, Verma et al. 2013). A mean PIC of 0.49 (Table. 3.6) in this study is almost half of 0.89 that was reported in a similar study on finger millet (Arya, Verma et al. 2013). The low PIC in this study can be attributed to the conserved nature of the coding sequences or it could also reflect upon the genetic diversity (Cordeiro, Casu et al. 2001).

Further analysis for these marker sets (31 markers) on ICRISAT population grouped these accessions into distinct clusters (Fig 3.5) showing relationships on the basis of allele sharing. Accession IE 4709 from Burundi was shown to be the most distinct. I expected the accessions to cluster according to their geographic origins. However it was seen for example that several sub-

clusters of Zimbabwe accessions were spread throughout the dendrogram. However, the India and Nepal accessions tended to cluster together. Considering the origin of *E. coracana*, it can be assumed that their relatedness might be associated with geography. On the other hand, landraces originating from areas other than Africa and Asia could be products of more recent globalization and redistribution. Overall this tree shows that these markers show striking genetic variation in the finger millet varieties from different geographical origins.

3.4.2 SSR markers studied on wild species of *E. coracana*

The wild relatives of *E. coracana* were included in this study to assess the transferability of these markers to other species and subspecies. Of the 95 selected SSR markers (47 novel primer pairs and 48 published primer pairs), 15 novel primer pairs and four published primer pairs amplified both the wild species and the ICRISAT accessions.

All the 95 selected SSRs were used to see the transferability of these markers in the 5 wild relatives of *Eleusine* (Table. 3.3). 81 primer pairs (86.17%) gave clear amplification for some of the wild relatives of *Eleusine*, of which 92.59% exhibit polymorphism. PIC values for SSRs ranged from 0.195 (ugep 10, ugep 24, ugep 56 and ugep 107) to 0.835 (ECS 215) with an average 0.573. Our results show that transferability of SSRs within the same family is high.

3.5 Conclusion

To aid in further improving this crop, the current study has explored the genetic structure of finger millet within a population obtained from ICRISAT. In

this present work, 83 accessions of finger millet have been genotyped with 31 SSR markers revealing an average PIC of 0.494. Genotyping the five wild species of *Eleusine* with 81 SSR markers revealed an average PIC of 0.573. These average PIC values indicate that SSR markers can be used for the genotyping of large germplasm collections of finger millet which will aid in their characterization. Thus from the present investigation it is clear that amplification of unique/SSR sequences in DNA has lead to the identification of distinct genetic identities for finger millet belonging to separate geographical origins. The identified SSRs can be used towards diversity and genome analysis. Exploration and evaluation of the entire available germplasm of finger millet will help identify superior lines based on various agronomical traits for breeding programs.

3.6 References

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3.7 Appendix

Appendix 3.7.1: Geographic origin of various finger millet accessions obtained from ICRISAT

Accession no.	Geographic origin	Abbreviated name	Accession no.	Geographic origin	Abbreviated name
IE 501	India	IND03	**IE 7320**	Kenya	KEN27
IE 518	India	IND12	**IE 2589**	USA	USA59
IE 2034	India	IND04	**IE 2606**	Malawi	MLW32
IE 2042	India	IND14	**IE 2619**	Malawi	MLW33
IE 2217	India	IND20	**IE 2710**	Malawi	MLW31
IE 2296	India	IND21	**IE 2790**	Malawi	MLW34
IE 2312	India	IND19	**IE 2821**	Nepal	NEP39
IE 3045	India	IND15	**IE 5537**	Nepal	NEP40
IE 3077	India	IND13	**IE 5817**	Nepal	NEP38

IE 3104	India	IND10	**IE 5870**	Nepal	NEP37
IE 3470	India	IND05	**IE 6059**	Nepal	NEP36
IE 3475	India	IND16	**IE 6082**	Nepal	NEP42
IE 4671	India	IND11	**IE 6154**	Nepal	NEP43
IE 4734	India	IND17	**IE 6165**	Nepal	NEP41
IE 4757	India	IND09	**IE 6221**	Nepal	NEP44
IE 4816	India	IND22	**IE 2871**	Zambia	ZAM62
IE 5201	India	IND18	**IE 2872**	Zambia	ZAM61
IE 2043	India	IND08	**IE 2911**	Zambia	ZAM60
IE 3618	India	IND07	**IE 2957**	Germany	GER02
IE 4673	India	IND06	**IE 3317**	Zimbabwe	ZIM68
IE 2430	Kenya	KEN30	**IE 3391**	Zimbabwe	ZIM82
IE 2437	Kenya	KEN26	**IE 3392**	Zimbabwe	ZIM81
IE 2457	Kenya	KEN23	**IE 4329**	Zimbabwe	ZIM67

IE 2572	Kenya	KEN28	**IE 4491**	Zimbabwe	ZIM71
IE 5367	Kenya	KEN24	**IE 4497**	Zimbabwe	ZIM83
IE 7018	Kenya	KEN25	**IE 4545**	Zimbabwe	ZIM79
IE 7079	Kenya	KEN29	**IE 4565**	Zimbabwe	ZIM74
IE 4570	Zimbabwe	ZIM66	**IE 3952**	Uganda	UGA47
IE 4622	Zimbabwe	ZIM63	**IE 3973**	Uganda	UGA53
IE 4646	Zimbabwe	ZIM65	**IE 4028**	Uganda	UGA52
IE 5091	Zimbabwe	ZIM69	**IE 4057**	Uganda	UGA56
IE 5106	Zimbabwe	ZIM78	**IE 4073**	Uganda	UGA48
IE 5306	Zimbabwe	ZIM76	**IE 4121**	Uganda	UGA50
IE 6240	Zimbabwe	ZIM75	**IE 6421**	Uganda	UGA54
IE 6294	Zimbabwe	ZIM77	**IE 6473**	Uganda	UGA55
IE 6326	Zimbabwe	ZIM64	**IE 4709**	Burundi	BUR01
IE 6337	Zimbabwe	ZIM73	**IE 4797**	Maldives	MDV35

IE 6350	Zimbabwe	ZIM72	**IE 5066**	Senegal	SEN46
IE 4795	Zimbabwe	ZIM70	**IE 1055**	Unknown	UN258
IE 6514	Zimbabwe	ZIM80	**IE 3614**	Unknown	UN157
IE 3721	Uganda	UGA51	**IE 6537**	Nigeria	NIG45
IE 3945	Uganda	UGA49			

Appendix 3.7.2: Novel SSR primers synthesized using transcriptome assembly.

SSR Marker	Primer sequence	SSR motif	T_m value of primer(C°)	Amplicon size (bp)
ECS1001R	ATA CGG CTC AGT CCC ATA CG	$(CTT)_6$cagagtcaggtcaaggcct ccatcctcggcaggtgccgcatcatc agcgggggggagcgtcggcgtcggc gtcggcatcgtcgaccttctttga$(CTT)_6$	55.695	231
ECS1001F	GTG AAG TCA TCA TCG CCC TT			
ECS1002R	CAA ACA CAC ACA CAC ACA CAC A	$(TG)_{11}$cagta$(GT)_{10}$	54.985	206
ECS1002F	GAT GGA GGA AGC CTA CTA TGA A			
ECS1003R	AGA AGT CGG AAA ACG AAC GA	(ATC)6ccaaccaaccc(ACCA)5	54.9	99
ECS1003F	CCA AGC ACG ACG ACA TCT AC			
ECS1004R	GCC CAT CTG AAA CCC TAA CA	(GA)21	56.72	185

ECS1004F	GAG GAG AGA GGG AGA GCG TC			
ECS1005R	CCA CAA GCC CTC GTT CAC		55.275	99
ECS1005F	GTG GTT CAT GGG CAT CAT TC	(ACACCA)5		
ECS1006R	CAA CAA TAG CCC AAA TGC CT		55.165	102
ECS1006F	GAG GAG TGA CTG CAT CGT GA	(GA)16		
ECS1007R	GCT ATA ACC GGC ATT CCA GA		53.88	104
ECS1007F	GGG AGC AAT ATC CAA GCA AA	(ACTGAG)5		
ECS1008R	TTC CAT GGA GCC CAC TTA AC		55.65	109
ECS1008F	TTG AAG CCT ACT TGA CCG CT	(TCC)7(CT)14*		
ECS1009R	GGT CAG GTC AGG TCA GGT CA		58.245	99
ECS1009F	ACG GCC ACA CGA CTG AAG	(GACCT)5		
ECS1010R	GCT CGC TTA TTT CGG AAG TG		54.145	109
ECS1010F	GGG TCA AAC AAA TCC ACC AC	(CTT)10		
ECS1011R	GCC GTG GAA GTA GAG GAT CA	(CCA)8	56.75	99

ECS1011F	CAC CTA CCT CCG CCT CTT C			
ECS1012R	GCG TTG TTC GAT CAT TCA GA		53.875	205
ECS1012F	TGG CAA AAG ACT GAA GAG CA	(AATA)6		
ECS1013R	CGT CCA GAG TTA ACC ACC GT		54.23	139
ECS1013F	ATC CAA TCC AAT CCA ATC CA	(TCT)16		
ECS1014R	CAC AAC ACC CAG CAT AGC AT		55.835	102
ECS1014F	TAG ATC CGT CAA GGA CCA CC	(ATGCT)5		
ECS1015R	CTG CGT GTG CTT GAC AAT TT		54.085	104
ECS1015F	CTC CCC ATT CTT ATC CCC AT	(CT)15		
ECS1016R	CAA GCG TGA TTT TCC TGT GA		54.605	137
ECS1016F	TCT GGC TCA TTT CCT GTC CT	(AATCT)5		
ECS1017R	TCC TCC CTC AAC AAC AAC AA		54.815	123
ECS1017F	GGA ACT GAT GGT TGT CAG TAG C	(TGT)7		
ECS1018R	GCT GCG AAT AAA AGT CAA AGT CA	(TAA)7	54.405	134

ECS1018F	TTC CGA TGC CTC TGA TGA AC			
ECS1019R	CAG CAG GTC CTC CAG GGT		59.475	99
ECS1019F	AGG AGG AGG AGG AGG AGG AG	(AGG)7		
ECS1020R	CCA TCA GAC TGT CAG GTA AGG A		54.56	117
ECS1020F	TTG TTG TTG TTG TTG TTG TTG C	(TTG)7		
ECS1021R	AAC AAC AAC AAC AAC AAC AAC AA		54.485	100
ECS1021F	AGT TTC AGT ACT CGG CAG CA	(TTG)7		
ECS1022R	GTC GTG TCC TTC TTC GTG G		56.345	102
ECS1022F	AGA AGG AGC TCT TCG TGC C	(GCC)7		
ECS1023R	TTC TTT CTT CTT CTT CTT CTT CTT CTC		54.5	110
ECS1023F	CAG CGG CAG CTT GTG TTA T	(AGA)7		
ECS1024R	TTG TTG TTG TTG TTG TTG TTG TTT		52.49	99
ECS1024F	TCA GCA TTC TAC AAA GGA ATA TGA	(AAC)7		
ECS1025R	GAG GGA GGA CTT GAG CCG	(CGA)7	56.895	123

ECS1025F	GCC GGT GAA GCA GGT ATT C			
ECS1026R	CTA GGG TTT TAT GCT TCT CAC ACT		55.315	115
ECS1026F	AAC GGG GTG TGA TGG TAG AA	(CGG)7		
ECS1027R	GTG GTG GTG GTG GTG GTG		57.48	100
ECS1027F	CGT CGA GTC CAT GCT GTT C	(ACC)7		
ECS1028R	GTC GTC CTC CTC CTC CTC CT		59.51	103
ECS1028F	TTC TCC TCC ACT GCC GCT	(GGA)7		
ECS1029R	TCA CGA GCA CGA GAG AGA GA		56.465	100
ECS1029F	CTC GTC CAG AGC CAA GAA AC	(CT)18		
ECS1030R	AGG CCT GGC GTT TCC TCT		58.365	102
ECS1030F	AGT GGT ATG CCC GAG CTG	(GGC)7		
ECS1031R	GGT TCG ACT TCT CCC ACC AC		57.385	105
ECS1031F	CGA TGG AGT CGG AGT CGA G	(TCC)7		
ECS1032R	GTA CTG CAC TCG CCG GAT	(GT)11	55.195	99

ECS1032F	CTG GAT TGA TCA TCG TCC G			
ECS1033R	AGG TAG AGG CAG AAT CCG GT		57.115	101
ECS1033F	CAT CGT CCT CTC CAC CAA CT	(CAC)7		
ECS1034R	TCG AGG AGG AAG AAG ACG AG		55.495	99
ECS1034F	TGA TGA TGA GGA GGA GGA GG	(GAG)10		
ECS1035R	GTT CAA GTG GCT TTG GTG GT		55.935	172
ECS1035F	GAT GCA GAA GAA GGC AGT CC	(CCA)7		
ECS1036R	GTT CAA GTG GCT TTG GTG GT		55.935	99
ECS1036F	GAT GCA GAA GAA GGC AGT CC	(CCA)7		
ECS1037R	TGA CAG CAT CCT TGT TGC TC		55.29	101
ECS1037F	TAA CAT GTG TTC GTC GAG GC	(GA)19		
ECS1038R	AAG GAT CGT GCA AGT GAA GG		56.555	103
ECS1038F	GCA GCC ACC ACT ACT TCT CC	(AG)21		
ECS1039R	GAC CAA TGC AAC AAG GTG TG	(GTAT)6	54.485	106

ECS1039F	GCT TGA TTT CGC CTG TGT TT			
ECS1040R	CGT CAT GAT GAA ATG ACG ATG		53.315	99
ECS1040F	GTC GGA TTC GAG GTC ACA AA	(TAC)7		
ECS1041R	TCA CCT GTG TGA TGA TCT CCA T		56.225	114
ECS1041F	CAG AGA GAG AGA GAG AGA GAG GG	(AG)10		
ECS1042R	TGC CCT GCT GTC GAT CAA T		57.135	99
ECS1042F	TCT CTC TCT CTC TCT CTC TCT ACG TC	(TC)10		
ECS1043R	ACA TGT ATG TAG TGA TCA TGT TCG G		55.015	101
ECS1043F	GAG AGA GAG AGA GAG AGA GAA ATA ACC	(GA)10		
ECS1044R	GGA GCA TAT TAT TAT TCA TGG TGG		52.74	99
ECS1044F	CGC GCT GTT CTT TTT CCA	(CCTG)5		
ECS1045R	CGG ATC ACC ACG TCA TTA AA		54.235	101
ECS1045F	AGC ACC TTC ACA TGC ACC	(ATC)7		

ECS1046R	AGC TAC GGC CTC GAG AAG G		58.13	103
ECS1046F	CAG ATC CCG GAG ACT CAG C	(CCG)7		
ECS1047R	CAA ACA AGT CTC TGA CCG ACT G		55.375	107
ECS1047F	CAC GGA CGA CGC ATA ATC TT	(TCAG)5		
ECS1048R	TTC ATG GAA TGA ATC TTT TCC C		53.095	99
ECS1048F	GGG TAA TCC ACG CCT TCT TT	(GTCT)5		
ECS1049R	CCA CGA ACA GGT TCT GCC		57.32	111
ECS1049F	ATA GCG CCT CAC CTC TCT AGC	(TA)10		
ECS1050R	GAA CTC CAT TGC CAG TTG GT		54.905	99
ECS1050F	CTT CCT TTC CCA CAT CCG TA	(TA)14		
ECS1051R	GGG AGG GAG AGA GAG AGA GAA		56.025	101
ECS1051F	ATA TCA GGA GCG TTT CGC AC	(CCCA)5		
ECS1052R	CCG CTG AAA AAC GCA AGT		55.115	99
ECS1052F	CTC CAC CAC GTT CTG CAT C	(TC)10		

ECS1053R	ACT GTG CAG TTT TAC CGC CT		55.415	101
ECS1053F	TTC TCA GTT TTT CAA CCG CC	(AGGC)5		
ECS1054R	ACA ACA AAC TGC ACT GCC TG		55.325	108
ECS1054F	CTT GGT TGG TTG GTT GCT TT	(AGGC)5		
ECS1055R	CAC TTG AGG GGA TGG AAG AG		56.02	107
ECS1055F	AAG CAA TGT ACA GTA GAG ACA GAC AGA	(AGAC)5		
ECS1056R	TGT TGT TGT TGT TGT TGT TGT TG		54.235	99
ECS1056F	AAG GCA AAT GGT CGT CAG AG	(CAA)7		
ECS1057R	GAT GGA TGG ATC GGA GGA C		54.815	192
ECS1057F	CAA CCA ACC AAC CAA CCA AC	(CAAC)5		
ECS1058R	GGC ATG CAT CTC CAT CAA AT		53.295	99
ECS1058F	CTT TCC TAG TTA TTG GGC CG	(TA)10		
ECS1059R	CAA TGC TCC CTG CTT CTC TC	(GGAG)5	54.88	106

ECS1059F	AAA GTT TTG GGT GAA GTG CG			
ECS1060R	ACC CCA ACC CCA ACA AAC		56.905	99
ECS1060F	TCC TCT CCA CTC GTC TCC C	(GCTC)5		
ECS1061R	CCT TGA AAC TTG ACA TTT GTA CAT C		52.68	106
ECS1061F	TGA GAG CTG AAA AAG AAA GAA AGA	(AAGA)5		
ECS1062R	GGT GCT TGG TAG ACC TGG AA		55.755	103
ECS1062F	CTT GTA ACT GCT GGT TTG CG	(TC)10		
ECS1063R	GTC AAG CAG AAG GTG GGT TG		57.445	107
ECS1063F	CAC CTA CAC GCC CTC ACC TA	(GAGG)5		
ECS1064R	CCC CTC CTT TTT GTT TTG GT		53.94	99
ECS1064F	CCC ATC TGC TTC GAT CTC AT	(AGGC)5		
ECS1065R	GTG CCC GAA TCG AGT AAA AT		55.08	101
ECS1065F	TCT CTC TCT CTC TCT CTC TCA GAC TAC	(TC)10		
ECS1066R	GTT GGA CTT GGT GGC GTA G	(GA)10	57.265	9

ECS1066F	GAG AGA GAG AGA GAG AGA GAG GAG C			
ECS1067R	CAA CCC AAG ATC CAT TTC AA		51.99	101
ECS1067F	CCT TCT TCT TCT TCT TCT TCT TGG	(CTT)7		
ECS1068R	TCT TGT GCT TTG GGA TGG AT		55.635	104
ECS1068F	CCG TGT CTT CGG ACT CGT	(AT)10		
ECS1069R	AGG TTC ACC ACC ACC ACC T		59.16	101
ECS1069F	CTC CTC CTC CTC CTC CTC CT	(CTC)7		
ECS1070R	TAA AAT TCC CCA CCC TCC TC		54.115	108
ECS1070F	TCT CGA TTG CTT GCT TCT GA	(TGTT)5		
ECS1071R	GCT ACA CCC GTA GGA GCA CT		56.955	114
ECS1071F	CCC TCT GGG AAG TTG GTT TT	(AG)10		
ECS1072R	TGA AGC TGG TTC TCG TCG T		56.085	99
ECS1072F	CTC TCT CTC TCT CTC TCT CTC AGA A	(CT)10		
ECS1073R	AAC ATC ATC ACA TCA CCG GA	(TC)10	55.78	126

ECS1073F	TCT CTC TCT CTC TCT CTC TCA GCA			
ECS1074R	TAG CGC ACA GCT CGC TAA TA		57.145	99
ECS1074F	CCC ACA CAC ACA CAC ACA CA	(CA)10		
ECS1075R	ATC CTT GGA CCC GGC CTA		57.575	145
ECS1075F	ACC TTG GTC GTC TTC CCT TC	(GTC)7		
ECS1076R	CCC AAG CAC ATC ATG TAA GC		55.09	104
ECS1076F	GTC GTC CAC CAT GAA GCT G	(TGAT)5		
ECS1077R	GGG AGG GAA CCT GAT GAG A		56.32	101
ECS1077F	TTC CAT CTC CCT CTC CCT CT	(TCCA)5		
ECS1078R	ATC GGG ATG GCC TAC TCT CT		57.455	103
ECS1078F	GCT ACT GTG GGC TTC CTC TG	(GA)10		
ECS1079R	ACG TGA TCG ATC GCC TAT TC		55.4	163
ECS1079F	GTA CAC CCA ACC AAC CAA CC	(GGGA)5		
ECS1080R	CAA TGT TGC CCA GGA CCT AT	(GA)10	54.73	99

ECS1080F	TTC GGT AGC CAT TTC TCT GTC			
ECS1081R	CTC AAC GAA TTC CTT CCA GC		53.465	102
ECS1081F	TTT TGG GGT GGA GTT TCT TG	(TTA)7		
ECS1082R	ATC GGA GCA AAG ATC AAG GA		54.425	99
ECS1082F	GGG ACG ATG TGT TCG TCT TT	(GCGGGG)5		
ECS1083R	GGC GTG AAG AAC AAT GGA AA		54.9	102
ECS1083F	CTC GAA TCC TCC TCC TCC TC	(TCC)7		
ECS1084R	CCA TGG AAA GAA ACG AAG GA		53.36	99
ECS1084F	CAC CTT TCC ACC CGT TTG	(TCCA)5		
ECS1085R	CAT GAA TTA CCC TGT CGC CT		54.715	101
ECS1085F	ACA ATC AAT GAG ATG GGC GT	(GT)10		
ECS1086R	CGG TCA CCA TAA GTG ACG TG		55.82	103
ECS1086F	CTT TCC GTT CCA GCA CTA GC	(AAAAG)5		
ECS1087R	CTG AGG GAG CGT GAG CGT	(CAC)6	58.265	115

ECS1087F	CCT ACG TCG ACT CGC TTC TT			
ECS1088R	CAG TCT TTG CCC TTT CCT CT		54.41	99
ECS1088F	TGG AGG CTT TAA GTT GAT TGA GA	(GAT)6		
ECS1089R	GAA GTG GCA GCA GCA GAA AG		56.735	102
ECS1089F	TGT ACG GAC GAA GCA GAG G	(TTC)6		
ECS1090R	GCC GCT GAT GAT GAT GAT G		55.12	99
ECS1090F	GAC TAC ACG ATG GTG GAC TTC A	(ATC)6		
ECS1091R	TTA TTA GCT TGT TGT TGT TGT TGT TG		52.385	102
ECS1091F	TTG ACA ATA TGC AAA AGA GTG TC	(ACA)6		
ECS1092R	TGT GCT TAA TGA AAA CAA CGG		54.655	99
ECS1092F	AGG CTC AAA CCT TGT GGC AT	(GCC)6		
ECS1093R	TTG TTT TGT TTC TAT GTG TGT GTG		52.575	102
ECS1093F	TTG ATG GAA GAA GCA TGC AA	(GA)10		
ECS1094R	GAC TCA TCA TCA TCA TCA TCA CAG	(TGA)6	54.87	104

ECS1094F	GAC TGC TCC AGG GTC AAC TAA			
ECS1095R	AAG GTC AAG ACA ACA TAC AGC G		54.54	109
ECS1095F	CAT GAT CAG CAT CAA CGT CC	(TCA)6		
ECS1096R	GAA GCT CGC GAG GCT GTA G		57.97	99
ECS1096F	GTG CAA CCA CCA GAC GCT A	(CGC)6		
ECS1577R	AGC TGG CAA CTT CAA TGC TT		54.66	240
ECS1577F	CAC CTT GGA TCA CAA TCA CG	(TTG)6		
ECS1578R	ATC CAC CAC CTC CAT AAC CA		54.785	255
ECS1578F	GCA ACT GCA TTC TTC AAC GA	(TGG)6		
ECS1579R	TTG CAT CAG CTC ACT CCA TC		55.765	264
ECS1579F	CCG ACC GTA CCT GAA GAT GT	(CT)10		
ECS1580R	AAG TAG AGG AGC TCC AGG GG		55.68	277
ECS1580F	TTC AGA TGT TCA GCG AAA CG	(CGG)6		
ECS1581R	TCT GCA ACT GTG GCT GAA AC	(TG)9	54.985	276

ECS1581F	GGG AAA GCC TTC TTG GAA AC			
ECS1582R	CTC TCT AGG CCA AAG ATG CG		56.06	230
ECS1582F	GCT TCC TCT CCC TAC CAT CC	(TCC)6		
ECS1583R	TCT CCC TCA ACG TCT CTC GT		57.395	215
ECS1583F	GCA GGT CGT CTC TGA ACC TC	(GGA)6		
ECS1584R	CTA TCT TGT CCG CGT TCC AT		56.13	-
ECS1584F	TTA GCC TCC CCC TCT CTC TC	(CT)9		
ECS1585R	GCC TGT TGT GAT GTG TAC CG		55.645	243
ECS1585F	AGA TCA TCA TGA CGC TGC TG	(GCT)6		
ECS1586R	CCG GAT AAT CTT GGA CCA TC		53.095	256
ECS1586F	TTT GGG TCT GTC ACG TTT TG	(ATA)6		
ECS1587R	CAG AGG GAG CAG GAC TTG AG		56.485	267
ECS1587F	GAA GGC AGA GCA TCT TCC AC	(CAC)6		
ECS1588R	TCT GGA ACC ATC TTG GGA AG	(GAT)6	53.79	277

ECS1588F	TGC AAA GCA GAT CAT GAA GG			
ECS1589R	ATC TGC CGT CGT CTA CCA TC		55.825	279
ECS1589F	CTT GGA TGA GCT TGG AGG AG	(CTC)6		
ECS1590R	TAT CTC CGC CAT CCA ATA GC		55.36	180
ECS1590F	CCA CCA GAC AGA CGA GGA AT	(AGC)6		
ECS1591R	ACT CAC TGC AAG ACG ACA CG		57	240
ECS1591F	GGG ACG AGA TCA CGG AGT T	(GGA)6		
ECS1592R	CTA CGA CTT GCC TTC CTT CG		55.34	-
ECS1592F	GAG GAG CTT GTC CAG AAT CG	(CCA)6		
ECS1593R	CTG CTC CTC GTC AAG TGC TA		56.79	272
ECS1593F	CTC GTA ATT GGG CTG GTC CT	(CGC)6		
ECS1594R	GCT GGG TTT GTG CTC TTC TC		56.23	257
ECS1594F	TCC CCC ACG TAC TTT ACT CG	(AG)9		
ECS1595R	CAT TCC TGA CTG TGG TGG TG	(CTC)6	54.245	267

ECS1595F	GCG TGA AGA AAA GCG AAA TC			
ECS1596R	AAC CCT CCT CTA GCC AGC TC		56.84	279
ECS1596F	GTC CAC CTT GCA TTC CTG AT	(GAG)6		
ECS1597R	AAC CCT CCT CTA GCC AGC TC		56.84	276
ECS1597F	GTC CAC CTT GCA TTC CTG AT	(GAG)6		
ECS1598R	GAG CCA CCT TCA CGT AGA GC		57.185	318
ECS1598F	CAG CGA TAC AGG AGG AGG AG	(GCG)6		
ECS1599R	TCG GTC TTG AAG AAA ACG CT		55.4	226
ECS1599F	GAG CTT GAA GGT CTT GGT GC	(CTT)6		
ECS1600R	GAA GTT TTG CAG CTT CGA CC		55.655	-
ECS1600F	AGG GGT CAG TGT GAA TCT GG	(CCG)6		
ECS1601R	ACA GCA ACA TCA GCA ATG GA		56.4	244
ECS1601F	AGG TCC TCG CAG TCT TGG TA	(GCC)6		
ECS1602R	CAG AGG GAG CAG GAC TTG AG	(CAC)6	56.485	257

ECS1602F	GAA GGC AGA GCA TCT TCC AC			
ECS1603R	CAG AGG GAG CAG GAC TTG AG		56.485	267
ECS1603F	GAA GGC AGA GCA TCT TCC AC	(CAC)6		
ECS1604R	CAG AGG GAG CAG GAC TTG AG		56.485	278
ECS1604F	GAA GGC AGA GCA TCT TCC AC	(CAC)6		
ECS1605R	CTG TAG CCC TCC TCC TCC TC		57.69	275
ECS1605F	CCA ACG ACG AGG TCA AGA GT	(GAG)6		
ECS1606R	GGA GGA AGA AGC GAA GGC		56.205	233
ECS1606F	TGA CAG TTC TTC CCC ACC TC	(CCG)6		
ECS1607R	GCA AAT TCA AGC CAG AGG AG		55.635	232
ECS1607F	AAC GAG TCC AGC TTG TTG CT	(TGT)6		
ECS1608R	GAA AGG AGA ACC ACC GAT GA		55.69	-
ECS1608F	CCA GAT GGT GGC CTA GTG TT	(TCT)6		
ECS1609R	CAG CAG AAA AGA GGT GGT CC	(GCC)6	56.745	243

ECS1609F	GTC TCT TCT CGG CTC ACT GC			
ECS1610R	GCT TGC CAA GTC ACC AAG AT		55.475	257
ECS1610F	ATG CAA TCT AGC CCG TTC AG	(CTT)6		
ECS1611R	CCA AAA TGC CCC TGA AGT AA		53.825	269
ECS1611F	ACC GAT TTA GGT AAT CCG CC	(TTG)6		
ECS1612R	CAC CTC CTC CAT CTT CTC CA		55.765	278
ECS1612F	GAA GCA TCG TCT CCG TCG	(CGA)6		
ECS1613R	AGG AGA TCA ACG TGT GGG AG		55.22	184
ECS1613F	GAA CGT CTG GAT TTC TTC GC	(GGA)6		
ECS1614R	AGG AGA TCA ACG TGT GGG AG		55.22	225
ECS1614F	GAA CGT CTG GAT TTC TTC GC	(GGA)6		
ECS1615R	TCG ACG AGG AAG AAG AGG G		55.12	229
ECS1615F	CAA TGG AAC AGT GAC CAT GC	(CGC)6		
ECS1616R	TTT GTC CTC CTT GCT GAT GA	(GCG)6	54.705	-

ECS1616F	GTT GAC GAA GAT GAC CTG GG			
ECS1617R	ATC CTC TAT GCT CAC CGC AG		57.09	248
ECS1617F	CAA GTC GCA CTA GCC TCC TC	(CCG)6		
ECS1618R	ATC AGC CCA TGA TGT ACG CT		56.17	261
ECS1618F	CTT CTT CTT GCC AGA GCC C	(GCT)6		
ECS1619R	AGA GAG AGC GGA GGA AGG AG		56.76	269
ECS1619F	AAG TTT GTC CTT GCA GGT CG	(CGG)6		
ECS1620R	TCT CCC TCT CGA TCC TGC TA		54.415	277
ECS1620F	GGA TTA TTA TTG GCG GGG AT	(TAC)6		
ECS1621R	GTC TGT AGC AGG GTC GTC GT		57.54	189
ECS1621F	GAA GAG CTC GCC AAG TCA AC	(GCA)6		
ECS1622R	ACT CCA CCG AAA GTC ACC AC		56.225	183
ECS1622F	GCG TCC CAT CTC TTC TTC AG	(CGA)6		
ECS1623R	ACT CCA CCG AAA GTC ACC AC	(CGA)6	56.225	168

ECS1623F	GCG TCC CAT CTC TTC TTC AG			
ECS1624R	ACT CCA CCG AAA GTC ACC AC		56.225	-
ECS1624F	GCG TCC CAT CTC TTC TTC AG	(CGA)6		
ECS1625R	TGT CGT CGG TTC CTC TCT CT		56.515	248
ECS1625F	GCG AGA GAA AGG TCT GGT TG	(GA)9		
ECS1626R	CAC CAA CTC TGG GTC CTT GT		56.01	260
ECS1626F	AGC CAA AAG AAG TGG AAG CA	(CAA)6		
ECS1627R	GAG GAA GTT CTT GAG CGA CG		56.26	270
ECS1627F	GAG CTG AAG GAC CCC CAT	(AGC)6		
ECS1628R	CAA TCC AAT CCG ATC CAT TC		54.345	275
ECS1628F	TAC CTC GTG GAG GAC CAG TT	(GCG)6		
ECS1629R	GAT GCC TTC CTT CCC TTC TC		55.29	175
ECS1629F	ATG CAG CCT TTG TCA TCT CC			
ECS1630R	AAT AGG GAG GGC GAA GAC TC		55.16	227

ECS1630F	TCA GCA CCA CCT GAA TAG G		
ECS1631R	TGC TGT GCT CCT CTG TTG AC	55.115	245
ECS1631F	AAT TCC ATT CTC TCG CAT CG		
ECS1632R	TGT CAA AAA CCG GAT CCA AG	53.99	-
ECS1632F	TCA TGC TCC ATG AAG AGT GTG		
ECS1633R	TAT CTG CTT GTG CAG CTT CG	55.82	249
ECS1633F	GTT GGC TGC TCT GCT TAT CC		
ECS1634R	CTA TGT CGT GTC CCA TGT CG	53.705	262
ECS1634F	AAA CGC GAT GAA TTT TAA GCT C		
ECS1635R	TGA CAA GAG CAC ACC GAC TC	55.435	267
ECS1635F	AAA TTC GCA TCC TTG CTG AC		
ECS1636R	AAG ACG CTG GTG GAA ATA GC	56.215	276
ECS1636F	CCT CGA GTG GGG ATC CAG		
ECS1637R	TCA AAG TGA TGC GTC AGG TC	55.82	224

ECS1637F	ATC CCC ACC TAC GAG ATG C		
ECS1638R	AAG CCA TGG ATC CTT CCT TC	54.245	241
ECS1638F	AAG GCA ATC TCG AAT GCA AC		
ECS1639R	TGT TCT TGA TTG CAA ACT GAT G	52.56	244
ECS1639F	TTG CAT GTG TTG CTT TTT GC		
ECS1640R	GGC TCC ATG AAG AGC TTG AC	55.97	-
ECS1640F	TTC AGT GGT GAC GGA AGT TCT		
ECS1641R	GTA TCC ACC TGC ATG CCA AC	54.475	249
ECS1641F	CAA TTG ATG TCA TTG GGA CAA C		
ECS1642R	CGT GAT CCC TCT CCT CTC TG	54.475	262
ECS1642F	GCC TTT TGA TTG TTC AAC TCG		
ECS1643R	TGT CCC TCA CTC GTC TCC TC	57.18	269
ECS1643F	ATG GGG TTA GGG TTC GAG TC		
ECS1644R	CCG TGA GCC TCG AGT TTT AG	54.145	278

ECS1644F	ATG TTG ATA GCC GGA AAT GG		
ECS1645R	GAT GGC CAC TAG GGA TGT TG	55.2	150
ECS1645F	CCA CGA GGC CAT ACT GAA TAG		
ECS1646R	TGC TGG GTG AAA CCC TAG AC	56.195	206
ECS1646F	TCA TGC TAG CTT CAA CAC AAC C		
ECS1647R	CCT CGA TGG CCA TTA TCA AG	53.69	192
ECS1647F	TGC CAC AAC TGT CAA CAA AAG		
ECS1648R	ACC ATA ATA GGG CCG CTT G	54.975	-
ECS1648F	CTC CGA TAC AGG CGT AAA GG		
ECS1649R	TTG TTT GGA CGT TGG ATG TG	54.39	250
ECS1649F	TGT ACA CAA CAC CAC ACT GAT G		
ECS1650R	TTT TCT ACT GGT GGG CGA AG	56.01	265
ECS1650F	AGC TCT GCT TGG TGG AGA AG		
ECS1651R	ACC GAA ACC CTT GTC AGT TC	56.17	273

ECS1651F	AGT GCT AGC TTC CCA TCA GC		
ECS1652R	TCA TTG ATG AAT CCG ACG TG	53.09	275
ECS1652F	CGG TCA GCA TAT AAC GAA TGG		
ECS1653R	TCA ACA AGG TGA AGC AGA GC	55.485	400
ECS1653F	AGC TGC AGT TTC AGT GGA TTC		
ECS1654R	GGT ACG GAG ACA TCG ACA CC	55.715	215
ECS1654F	GCA CGT ACG GAT TCA CAT TG		
ECS1655R	CTC GTA AGC ACC CAC CTT TC	54.37	297
ECS1655F	TTC GCG CGA AAT ATA GGC		
ECS1656R	TAC AAC GTC CAG GCA ACA AG	54.01	-
ECS1656F	AAG CAA TCA ACA AAG CCT TTT C		
ECS1657R	CAC TCG AGA ACC GAC CTT TG	56.43	252
ECS1657F	AAG GGC CAT ACC AAC ACT CC		
ECS1658R	AGA CGC AAA TGG GTA AAT GTC	54.365	265

ECS1658F	ATT TCC GCC ATC ACT CCA C		
ECS1659R	CGA CTC CAG GTG TTG TTG G	56.28	272
ECS1659F	GGC CTT TGC AGT CAT GTG AG		
ECS1660R	CAG TCC AGG TTG GTT GC	52.84	275
ECS1660F	GCG AAA ACA CAA TGC AAA AAG		
ECS1661R	CCA GCG ACG GTA CCG CTT GTA GCG	65.245	192
ECS1661F	TCC TCC CTC CCT TCG CCC ACT		
ECS1662R	CCG GAA GCC GTG TAC TAG GGG AC	63.03	226
ECS1662F	ATC AGC AGC CAT GGC AGC GAC		
ECS1663R	GTT TCC GCA TGC ATA GAG CA	56.38	232
ECS1663F	AAC GCG AGG ACA CGT ACT TAC		
ECS1664R	CCT CGC CAG CGT CCT CCT CC	67.16	-
ECS1664F	ACC CTC TCC GCC TCG CCT CCT CCT		
ECS1665R	GTC CGT TCA CGT CCA ACT	55.905	255

ECS1665F	GCC TCG AGC ATC ATC ATC AGA		
ECS1666R	GGG AAT CAA TCC TGG CTA	54.075	265
ECS1666F	CCG GCG ATA AAA CAA TGA GGC		
ECS1667R	CAA CGA AAG TGG TTT ATG TG	49.515	274
ECS1667F	TTC CCT GTT AAG AGA GAA ATC		
ECS1668R	CTC CTC CGG CCG GTC TTC GAC TG	64.6	275
ECS1668F	CTC TGT CTC CTC CCC CGC GTC		
ECS1669R	GTA GTC CTT CTT GTA GA	48.775	153
ECS1669F	CTT TGT CTA TCT CAA GAC ACT TGC		
ECS1670R	GCA GAA GAG TAC GTA CCC CCG CG	62.2	162
ECS1670F	CGA TCC ATT CCT GCT GCT CGC		
ECS1671R	ACG ATC TAG TCT CTA AGG GGA G	55.245	-
ECS1671F	CTA CTC ATC AAC CGC ACA CG		
ECS1672R	TCC CTC CCT CCG TTT TCT C	57.225	-

ECS1672F	AGA CGA GAC GAA GTG GTA GTG G			
ECS1097R	GAG GTC CTC GAG TCC AAG GT		57.24	99
ECS1097F	TCC ATA GAA CCA CCA CAG ATC C	(CAA)6		
ECS1098R	CTT GAT AAT GAA TGG AGG CAC A		53.055	99
ECS1098F	GTA TCA GAT TTT CCT GGC CC	(TC)9		
ECS1099R	CCT GTT TTT CTG ATC ACG GG		54.29	99
ECS1099F	AAG CTA AGC ATC CAT CCA TCC	(TGA)6		
ECS1100R	GGA ACT ATA TAT GCC CAC CCT TT		55.03	100
ECS1100F	GAG AGA GAG AGA GAG AGA AAA CCC	(GA)9		
ECS1101R	GAA GAA CAA AGC GGT GGG AG		56.545	101
ECS1101F	AGG TAT ATG TCG ACA GCG GC	(GCG)6		
ECS1102R	CTT TCA GCT CCA CGG CCT		56.98	102
ECS1102F	CTG CGT CGG AGC CAT TAC	(GGC)6		
ECS1103R	TGT GGG AGT GTT CAA TCA AGT C	(AC)9	53.695	102

ECS1103F	TAA AAT GTG GCC CAT TCC AT			
ECS1104R	CTG CTG CTG GTG CTG CTT		58.245	104
ECS1104F	CTC TCT CTC TCT CTC TCT CGG C	(CT)9		
ECS1105R	TGC CTC AAC GCC TCT CTC		57.255	99
ECS1105F	GCT CCT TTC TCT CTC TCT CTC TCT C	(TC)9		
ECS1106R	CTG GTG GTG CCT CCT TGT T		55.655	99
ECS1106F	CTT GTA TCT TCT TCT TCT TCT TCT TGG	(TCT)6		
ECS1107R	CTC GGC TGG CTT TTG GTG		55.83	99
ECS1107F	ATT AAA ATG AGA GAG TCT GAT TAG CCC	(CAC)6		
ECS1108R	ATG CTG TTG CTG CTG CTG		56.54	100
ECS1108F	ACC TCG ACC GAT TCC TCC	(AGC)6		
ECS1109R	TTG AAT ATA TAG GCC GTG TCT CC		55.985	101
ECS1109F	TCT CTC TCT CTC TCT CTC CCT CC	(TC)9		

ECS1110R	GGT GGT TGC ACA CGT AGA GG		56.485	102
ECS1110F	ATC GCC GGT CCT TTT TCA T	(CAA)6		
ECS1111R	CAT GAT GAT CAG AAC TCT CTT ACT		53.62	103
ECS1111F	AGC			
	GAT CAT CCA TCC ATC CAT CC	(CATC)5		
ECS1112R	CAC ACG TTT GTG AAG GCA AC		54.745	174
ECS1112F	CGT CAG GCC GAT TGT AAG TA	(TTG)6		
ECS1113R	CCA CCA CCT GGT TCC TGT		55.06	99
ECS1113F	TCT TCT TCT TCT TTT TCT TCT TCT TCA	(TCT)6		
ECS1114R	CGC TTG CTC TAT TGA CAC CA		55.47	110
ECS1114F	GTC GTC GAC TTC CTT GAA CG	(TTG)6		
ECS1115R	CGT TAG TAC TAG CTC CGC CG		57.435	133
ECS1115F	ACT ACA ACA ACC GCG ACG AC	(GCG)6		
ECS1116R	AAG CAC GGC AGG TCG AAG	(CCT)6	56.805	117

ECS1116F	CAT CCT TGG CTT GAC TCC TC			
ECS1117R	GTT GAG TGG GAT GCC CTT AG		55.985	101
ECS1117F	CAC CTA CAC ATA GAA GTG TCG CA	(TA)10		
ECS1118R	CTC GTG GTG GTG GTG GTG		57.62	102
ECS1118F	CAT GCG CCA GCT GAA CTA C	(CAC)6		
ECS1119R	GAG CGA CTC CTC CGC ATC		57.545	103
ECS1119F	CGT GGG TGG GCT TGT TTC	(GTC)6		
ECS1120R	ACA ATC TCC ACC ACC ACC AC		57.185	146
ECS1120F	AGC GTC ACC TTG TTC CCT TT	(GTG)6		
ECS1121R	TGC CAT TTT GTT GTA CAC TGC		55	99
ECS1121F	TGC AAA CAC ACA CAC ACA CA	(AC)9		
ECS1122R	TTC TGG TTG CTG TTG CTG TC		56.85	99
ECS1122F	ACC AGC CCT CTG ATC CTT CT	(CGG)6		
ECS1123R	TAA TAC GCA ATG AAG CGC AG	(TTC)8	52.735	133

ECS1123F	CCA TGA TGG CCA AAT AAA CC			
ECS1124R	CAT CCT CTT CTT CTC CAG CG		54.695	112
ECS1124F	GAT CTG TGG TCG GAT TGG AT	(GTCG)6		
ECS1125R	ATT TCT CGG ATC CCC AGC		56.805	118
ECS1125F	GTA CGT GCC ACC TCC TCC T	(CCT)6		
ECS1126R	CTT GGA GAC GAG GGG GTC		56.5	102
ECS1126F	CCG AAT CCT CCT CCT CCT	(TCC)6		
ECS1127R	TGA CTG TTG TAA CAG AGA GAG AAG C		56.34	144
ECS1127F	GAG GGA GAG AGA GAG AGA GAG AAA	(GA)9		
ECS1128R	CAG ATC AGA TCC GAT GAG GTG		55.895	105
ECS1128F	CTC TCT CTC TCT CTC TCT CGC TC	(CT)9		
ECS1129R	CAT TAC CGT CGT CCT TTT CTT C		56.23	99
ECS1129F	GTG CGA GTA CCA GCA CCG	(GCA)6		
ECS1130R	TGA CCA CAA TCT TTC TGG GG	(AGGG)5	54.18	99

ECS1130F	TTC TCA AGC TCA TGT GCA AAG			
ECS1131R	GCC ATG GTT GCT GTC TTT TT		54.98	100
ECS1131F	GCA CAT GAC CCG AGA TCC	(TAA)6		
ECS1132R	ACG TAC TGG TAC GAG CCC A		56.65	101
ECS1132F	AAC GGC ATT GTG AGG AGG	(AGC)6		
ECS1133R	CTC ATG GAG ACT CCT ACC GC		57.49	101
ECS1133F	GCA GCT GCC TGT AGT TCT CC	(TCA)6		
ECS1134R	TCG CAA AAC TCA CAG ACA GC		55.55	102
ECS1134F	CTT GGG AGG AGA ACA ACC TG	(CGC)6		
ECS1135R	CTT CTG CAC CAG CAT CCC		57.85	121
ECS1135F	GAG CAC GAG CAG CAG CAG	(AGC)7		
ECS1136R	CTC TCT GGC TTT TTG CCT TG		56.01	105
ECS1136F	ACT ACC ACC GAG TGC TTT GC	(GATC)5		
ECS1137R	CCA TCC GCA TCT CTC TCT CT	(GA)9	56.395	99

ECS1137F	GGA ACC AGC TCT ACA TGC CT			
ECS1138R	TGC ATG TTT CTG TTA AAT GCC		52.79	99
ECS1138F	GCA ATG TCT ACC AGA TGA TGA TG	(GAT)6		
ECS1139R	CGC TAC ATC ATT CCT AGT CAT CC		55.29	100
ECS1139F	AGA GAG AGA GAG AGA GAG ATG TTC A	(AG)9		
ECS1140R	GAC GAG GTT GTA CGC GGG		58.485	101
ECS1140F	GAC GAG TCC GAC GTC TCC T	(CCG)6		
ECS1141R	ACC GAG ATG GCC CTA GAA AA		55.96	101
ECS1141F	TGC TTA GCA AGC CAT GTA GAG A	(AG)9		
ECS1142R	CCC ACA AAC AAC AAC AAC AA		54.665	102
ECS1142F	CAG CTC GTC CAG CTC CAT	(TGT)6		
ECS1143R	GTG GAG ATT CCT CCT CCT CC		56.435	208
ECS1143F	CTC TTT CCT GGG CCT CTT CT	(AGG)6		
ECS1144R	ACC AGC TCG TCG CGT ACC	(CAA)6	59.12	105

ECS1144F	CCA ACC TCA CAG CAG ACC AG			
ECS1145R	TTT GGG CAT TTC TTG CTT GT		55.515	99
ECS1145F	CAC AGA GAG AGA GAG AGA GAG CAG	(AG)9		
ECS1146R	GCA CCA CTC CAC CAC CAC		58.515	99
ECS1146F	AGA GAC AAC CGC ACC AGC	(AGA)6		
ECS1147R	AGT CAC TCA CCC AAA CAC CC		57.46	100
ECS1147F	GGT CAG TGA GGT TGG TAG GC	(CGG)6		
ECS1148R	TTG CTC GAG GAG GTA CAT GA		54.38	101
ECS1148F	TTG TTG TTG TTG TTG TTG TTG C	(TTG)7		
ECS1149R	GGA AGG GAA GGG AAT GAA AG		54.905	101
ECS1149F	CGA GGC AGG GTA ATC CAG T	(GCG)6		
ECS1150R	TTG AAG TGC AAA ATG AAA TGA TG		50.98	102
ECS1150F	CAT AAG ATA AAT ATT GTG CGG CT	(CAT)6		
ECS1151R	AGA AGG GCG AGA GCA AAG AG	(CA)9	57.24	104

ECS1151F	AAC CAC ACA CAC ACA CAC ACA			
ECS1152R	ACT TGC ACA TGT TCT TGC CA		53.36	105
ECS1152F	TAT CCC AAT TCC CAA ATC CA	(TC)16		
ECS1153R	GAA CGC ATT TCT TCC GTG AT		54.25	99
ECS1153F	GAT CCT GAA CCT GCA ACG AT	(TTAT)5		
ECS1154R	TCG ATT CAT ATT CAA TGA CAA ACA		50.945	99
ECS1154F	TGA TTT GTT GAA TGG AAA GGT T	(AT)10		
ECS1155R	ACA AGA ACG CCT TCA CCA AC		55.675	100
ECS1155F	TCT TGT CGA CGT TGT GCT TC	(TTG)7		
ECS1156R	CCT GGT GGT ACT GGA TGC TT		56.94	101
ECS1156F	CTT CCC ATG GTA GTG GCA GT	(AAG)9		
ECS1157R	CAG ATG AAG AGC TCC GTT CC		56.245	181
ECS1157F	GAG AGA GGC GAG GTC ACA AG	(GGC)6		
ECS1158R	GTT GCT CAA GCC TCA ACT CC	(TCA)6	56.51	102

ECS1158F	CCT CTT CAG TCT CCT CAC CG			
ECS1159R	GCA GCT TGT TGT CGT TGT TT		55.765	104
ECS1159F	GGG ATC TCG AGG GCC ATT	(AAT)6		
ECS1160R	TCC CTC ACA ACA ACA ACA ACA		54.33	105
ECS1160F	AAA ATA TCA TGG CAG GTG TGC	(TGT)6		
ECS1161R	TAT GCC CAT CAT CTT CCT CC		55.01	99
ECS1161F	GGC CAC TCC ATC ATC TGC	(GAG)6		
ECS1162R	AGA AGG CTC CGG ATA TGA TG		53.585	110
ECS1162F	GGT TGT TGT TGT TGT TGT TGG	(GTT)6		
ECS1163R	GCA GGA GGC ACA GCT CTC T		57.955	100
ECS1163F	TCT GCC AGA GCT CAT AGT ACA AC	(GA)9		
ECS1164R	CAG TTG CCC ATG CTC TCC		56.71	101
ECS1164F	CTC TCG TCC CTC CAG CTT C	(AG)9		
ECS1165R	CAG ACA CAC ACA CAC ACA CAC A	(TG)9	56.42	113

ECS1165F	GGT GGT TCA TTC GCT ACC AT			
ECS1166R	GAT TTC CCT CCC TCA GCA G		55.095	102
ECS1166F	AAG GAG AAG GAG AAG GGG AA	(CTG)7		
ECS1167R	GTG ATC CGA ACA ACC CTC TC		55.57	104
ECS1167F	CAC ACG GCC AGA CAA AGT T	(GA)9		
ECS1168R	CTG TTG CTG CTT CCC CTG		57.7	105
ECS1168F	CCA CCA GCT GTC CTC CTC T	(CTC)6		
ECS1169R	TCC CCC TTC CTC TCT CTC TC		54.43	99
ECS1169F	TCC CAA AAG AAA TCC ATC CA	(GA)9		
ECS1170R	ACC TCT TCC CGT CCC CTT		58.09	99
ECS1170F	GTC GAA GTC CGC GAG TGA G	(CCT)6		
ECS1171R	ATT GCC ATT GGT GGT GGT		53.725	111
ECS1171F	CAT CAT CAA ATC ATG CAG CAG	(CCA)6		
ECS1172R	CTC CAC ACC GAC CAG CTT A	(TGTCG)5	55.24	101

ECS1172F	CCT ATC AAC AAA AGC ACG CA			
ECS1173R	CTG CTG TGG TGC TCA GGT C		57.91	113
ECS1173F	GTC CTC TTC TCT GCG ACG AC	(CGG)7		
ECS1174R	AAA CAT TGC TGG AGT GGA CC		56.21	119
ECS1174F	TCT CTG CAC AGG CCA TAC TG	(CT)18		
ECS1175R	CCG GAG ATT CAT CTT CCT CC		54.785	104
ECS1175F	ACT TCC TCG CCG AAA TCC	(CAA)6		
ECS1176R	ATG CGA CGA AGG GAG TGA T		55.755	105
ECS1176F	AAA CAA CCC TAA GCT CAC CG	(CGC)6		
ECS1177R	TAC TCG AAC CCC TTC TCG TC		56.745	99
ECS1177F	GGC GAC AGA GTA GAA GAC GC	(CGG)6		
ECS1178R	TTT GGC TGC TCT TGA AAT CC		54.125	99
ECS1178F	CCC TTC TTC TTG GTG GTG AA	(CTT)6		
ECS1179R	GCC ATC TCC ATG TCC TCG	(GGA)6	56.475	100

ECS1179F	GTC CAC AAA GCT GTG GAG GT			
ECS1180R	CCC TTT ATG CCA CTG CTC AA		55.34	101
ECS1180F	AGC TAG ATT GCT TGC TTC CG	(CGG)6		
ECS1181R	CAA GGT CAC TCG TGT CTC CC		57.11	119
ECS1181F	GCA GGC AGC TGT ACA CAA AG	(AG)9		
ECS1182R	GTT ACG CAG ACG AGC ATC AG		56.605	102
ECS1182F	GCG GCT GGA GTA GTT GTG AT	(GTTTGA)5		
ECS1183R	TGC TGC TCT CGA GCA TCT TA		54.83	104
ECS1183F	CGG GCA TTC AAA CAA GTT CT	(TGCTG)5		
ECS1184R	GCA TCC TGT ATT AAT TTT CAC GC		55.705	106
ECS1184F	GCG TGT GTG TGT GTG TGT GT	(GT)9		
ECS1185R	AGT ACT TCA CCA TGG CTG CG		56.565	99
ECS1185F	CGT TCA GCC GTC ACT TCT TT	(CCG)6		
ECS1186R	GCT TCG CAA GAA ATT ATC AAT C	(AG)9	53.805	99

ECS1186F	TTT CTG TAG CCT GTG GAG CC			
ECS1187R	GGA GAT CCT CCT CTC ACT CTC A		57.41	100
ECS1187F	TGA CAA CAG CTC CTC CTC CT	(CTC)6		
ECS1188R	CCC TAC TCG TCT CCA CGA CA		57.78	101
ECS1188F	GAG GTC CAG GTT GAG GTC G	(TTG)6		
ECS1189R	GAC ACG CTG CTG CTG GAC		58.985	153
ECS1189F	AGT CCT TGC CGT CCT CCT T	(CGT)6		
ECS1190R	AAG AAC CCC CAG CTT TGT G		55.685	102
ECS1190F	GCT GGC CGT TAT TGC TGT TA	(TTA)6		
ECS1191R	CCC ATG GCT AGC TGT TGT TT		54.575	104
ECS1191F	TGA TTC CAT CAC CGC ATC TA	(CAT)6		
ECS1192R	CCC ATG GCT AGC TGT TGT TT		54.575	106
ECS1192F	TGA TTC CAT CAC CGC ATC TA	(CAT)6		

Chapter 4 – Concluding remarks

4.1 Physiological responses of finger millet to water withholding

Chapter 2 of my thesis outlines the physiological responses exhibited by finger millet (*Eleusine coracana* (L.) Gaertn) when subjected to water stress. Two previous studies (Bhatt, Negi et al. 2011; Parvathi, Nataraja et al. 2012), in finger millet have investigated the effect of drought on photosynthetic parameters, enzymes, chlorophyll components and metabolite accumulation. An elaborate study on relations of the above mentioned parameters with respect to plant's relative water content was not determined. By subjecting finger millet to water stress by withholding water supply, we demonstrated that these plants perceive and respond to stress. Based on all the 12 parameters studied, it was concluded that IE 7079 from Kenya was the most sensitive accession and IE 5091 from Zimbabwe was the most tolerant accession.

4.2 Screening for polymorphic SSR and defining finger millet germplasm

Chapter 3 of my thesis describes the identification of simple sequence repeat (SSR) from transcript assemblies of four RNA samples of finger millet obtained from ICRISAT. Previously described SSR markers have been used to create linkage map of finger millet (Dida, Ramakrishnan et al. 2007) and score finger millets based on their protein content (Kumar, Sharma et al. 2012). The 47 novel primer pairs validated in my study was used to define the genetic diversity of the finger millet population obtained from ICRISAT. Cluster analysis showed

that the 32 identified polymorphic SSR primer pairs show distinguishable variation in the ICRISAT accessions from various geographic origins. With the addition of these 32 novel SSRs of finger millet, to the existing genomic and genic SSRs (Dida, Ramakrishnan et al. 2007; Kumar, Sharma et al. 2012; Arya, Verma et al. 2013), it increase the potential for crop improvement and fundamental discoveries by identifying superior lines for finger millet which can be used for breeding programs.

4.3 Future perspectives

The work presented in Chapter 2 revealed some of the physiological responses of finger millet to water withholding. Chapter 3 described the identification of polymorphic SSRs in finger millet. A large number of responses occur both at the physiological and molecular level of a plant when subjected to drought (Farooq, Wahid et al. 2009). From my observations on the physiological responses of finger millet to water stress, it will be interesting to see the expression patterns of those plants that were able to withstand the stress. It could lead to the identification of novel genes, as there were other stress responsive genes reported (Parvathi, Nataraja et al. 2012).

The analysis of USDA and ICRISAT lines has not lead to a clear understanding of inheritance pattern in finger millet accessions. It will also be interesting to further analyze the data to understand the ancestory relationship. Using sequences from the nuclear and plastid DNA has known to be useful for studying phylogenetic relationships (Neves, Swire-Clark et al. 2005). Further, the

identification of 32 polymorphic SSRs in this study can be used to improve the existing linkage map of finger millet (Dida, Ramakrishnan et al. 2007).

4.4 References

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