# MARKER –ASSISTED INTROGRESSION OF STOVER QUALITY QTL IN PEARL MILLET

BY

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THESIS SUBMITTED TO THE ACHARYA N.G.RANGA AGRICULTURAL UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

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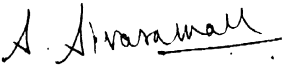
## CERTIFICATE

Ms. N. SRIDEVI has satisfactorily prosecuted the course of research and that the thesis entitled "**MARKER ASSITED INTROGRESSION OF STOVER QUALITY QTL IN PEARL MILLET**" submitted is the result of original research work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or any part there of has not been previously submitted by her for a degree of any university.



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Chairman of the Advisory Committee

**Date :**29th January, 2005

**Place :** Hyderabad.

## CERTIFICATE

This is to certify that the thesis entitled ***"Marker Assisted Introgression of Stover Quality QTL in Pearl Millet"*** is submitted in partial fulfillment of the requirements for the degree of **"MASTER OF SCIENCE IN AGRICULTURE"** of the **Acharya N.G.Ranga Agricultural University, Hyderabad,** is a record of the bonafide research work carried out by Ms. N. SRIDEVI under our guidance and supervision. The subject of the thesis has been approved by the student's advisory committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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## DECLARATION

I, **N. SRIDEVI,** hereby declare that the thesis entitled **"MARKER ASSISTED INTROGRESSION OF STOVER QUALITY QTL IN PEARL MILLET"**, submitted to **ANGRAU** for the degree of **MASTER OF SCIENCE IN AGRICULTURE**, is a result of original work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

**(N. SRIDEVI)**

**Date:** 29th January, 2005.

**Place:** Hyderabad.

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## ABBREVIATIONS

| | |
|---|---|
| °C | : degree Celsius |
| µl | : microlitre |
| µg | : microgram |
| AFLP | : Amplified Fragment Length Polymorphism |
| BAC | : Bacterial Artificial Chromosome |
| BC | : Back Cross |
| bp | : base pair |
| cM | : centi Morgan |
| CTAB | : Cetyl Trimethyl Ammonium Bromide |
| CMIE | : Centre for Monitoring Indian Economy |
| DNA | : Deoxyribo Nucleic Acid |
| dNTP | : deoxy Nucleotide Tri-Phosphate |
| EDTA | : Ethylene Diamine Tetra Acetic acid |
| g DNA | : genomic DNA |
| IAA | : Iso-amyl Alcohol |
| LG | : Linkage Group |
| M ha | : Million hectares |
| M | : Molar |
| MAB | : Marker Assisted Breeding/Backcrossing |
| MAS | : Marker Assisted Selection |
| Mb | : Million bases |
| ml | : millilitre |

| | |
|---|---|
| Mm | : milliMolar |
| ng | : nanogram |
| PAGE | : Poly Acrylamide Gel Electrophoresis |
| PCR | : Polymerase Chain Reaction |
| pmol | : picomole |
| QTL | : Quantitative Trait Loci |
| RAPD | : Random Amplified Polymorphic DNA |
| RFLP | : Restriction Fragment Length Polymorphism |
| RE | : Relative Efficiency |
| RIL | : Recombinant Inbred Line |
| RIP | : Recombinant Inbred Population |
| RNA | : Ribo Nucleic Acid |
| Rnase | : Ribonuclease |
| RP | : Recurrent Parent |
| SCAR | : Sequence Characterized Amplified Region |
| SNPs | : Single Nucleotide Polymorphism |
| SSR | : Single/Simple Sequence Repeat |
| TAE | : Tris Acetic EDTA |
| TBE | : Tris Borate EDTA |
| TE | : Tris- EDTA |
| TEMED | : N,N,N,N-Tetramethylethylenediamine |
| UV | : Ultraviolet |
| V | : Volt |

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## ABSTRACT

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important staple crop of the semi-arid regions of India and Africa. It is a dual-purpose crop grown both for its grain and fodder/stover. Crop residues provide the bulk of the livestock feed across South Asia, but their nutritive value is so low that farmers must supplement these with feed grains and other concentrates. Improving the nutritive value of the straw/stover and the efficiency of their use in mixed diets is an important option for increasing livestock production in the region. Genetic variation in the quality of pearl millet stover can be exploited to develop improved crop germplasm with stover of high nutritive value and good digestibility. In this context, marker-aided selection is found an ideal approach to transfer stover quality traits to elite genetic backgrounds through backcrossing with minimum linkage drag. To exercise marker-aided selection, a well-saturated molecular marker linkage map and tightly linked markers are a prerequisite. The first molecular-marker-based genetic linkage map of pearl millet was generated by Liu *et al.* (1994). That

map consisted of 181 RFLP markers covering the 7 pearl millet chromosomes and spanning a genetic distance of a 303 cM, and has since been extended with AFLP and SSR markers (Breese *et al.*, 2002; Qi *et al.*, submitted). A subset of these markers has subsequently been transferred to a series of different crosses that segregate for agronomically important traits. Quantitative trait loci have been mapped for downy mildew resistance (Jones *et al.*, 1995, 2002), drought tolerance and other genotype × environment interactions of grain and stover yield (Yadav *et al.*, 2002, 2003, 2004) and for characters involved in domestication (Poncet *et al.*, 2000, 2002).

ICRISAT and ILRI Scientists (Hash et al., 2003) have attempted to map the QTL associated with stover quality as well as grain and stover yield and aspects of drought tolerance using the RFLP- and SSR-based linkage map of ICMB 841 × 863B. Testcross hybrids of 79 progenies from this population were evaluated for stover traits at ICRISAT, Patancheru. Stem sheath and blade fractions of stover samples taken from different parts of the plant were evaluated for a number of *in vitro* estimates of ruminant nutritional quality. Subsequent QTL analysis detected a putative major QTL for leaf blade quality from parental line 863B (Hash *et al.*, 2003). This putative QTL has subsequently been partially introgressed into the genetic background of the more elite parent 841B = ICMB 841 by marker-assisted backcrossing.

Genetic linkage maps have been developed in various pearl millet crosses and used to detect and map quantitative trait loci (QTLs) contributing to various traits including stover quality. Information on the position of QTLs relative to marker loci

provides a basis for marker assisted selection (MAS) for quantitative traits. In crops like pearl millet, barley etc MAS is of particular interest for the development of genotypes stover quality, grain and malt quality etc because (1) thorough assessment of grain yield and quality traits is expensive and requires larger grain samples than are normally available in the early stages of a breeding program. (2) grain yield and quality traits are subject to considerable environmental variation and genotype x environment interaction.

With MAS for QTLs that affect grain and stover quality, pearl millet breeders could limit breeding populations to those progeny with the highest probability of having superior stover quality. Our objective is to assess whether marker-based selection could be effective in manipulating a QTL region in pearl millet breeding population in which the QTL region had originally been detected and mapped.

# CHAPTER #1

# INTRODUCTION

# CHAPTER I

# INTRODUCTION

Pearl millet [*Pennisetum glaucum* (L.) R. Br.], is the 7th most important cereal crop grown globally and the 2nd most important cereal crop grown primarily for dual-purpose use (grain and fodder/stover) after sorghum. It is an important staple crop of the semi-arid regions of India and Africa. Pearl millet is primarily grown as a rainfed crop in the low rainfall zones of Sub-Saharan Africa and the Indian subcontinent. This crop is grown as part of smallholder crop-live stock production systems in the sub-humid, semi-arid and arid tropics and subtropics where most of the worlds poorest livestock producers and consumers are found. In India, the average annual area sown to pearl millet amounts to 9.5 M ha with an average annual grain production of 8.3 M ton and average grain yield of 880 kg/ha (CMIE, Feb, 2004).

Pearl millet is an important crop in plant genetic research due to its dual-purpose nature, its adaption to adverse climatic environments, and the diverse range of germplasm that has been collected and is available for exploitation to the plant breeders. The cultivated crop and its wild progenitor are diploid with seven pairs of large chromosomes and a haploid DNA content of 2.4pg. The genome size of pearl millet (*P. glaucum*) is around 2300 million base pairs of DNA, which is much larger and about 5 times than that of rice (430 M bp) and almost equal to the size of maize genome (2400 M bp). The genome size of pearl millet is also larger when compared to sorghum (750 M bp). (Arumuganathan and Earle, 1991).

Because of the dual-purpose nature of pearl millet and its adaptation to adverse climatic conditions, where livestock products often provide rural families with much of their cash income, stover quality is of increasing importance. Improving the nutritive value of the stover and the efficiency of its use in mixed diets is an important option for increasing the livestock productivity of any region. The word 'stover' refers to dry leaf and stem residues fed to livestock following harvest of grain crops. Although such crop residues provide the bulk of livestock feed across the world, their nutritive value is low so that farmers need to supplement them with feed grains and other concentrates. Genetic variation in pearl millet is being explored to develop improved crop germplasm with stover of higher nutritive value and good digestibility. Stover quality is reported to be controlled by Quantitative Trait Loci (QTL) (Hash *et al.*, 2003). Hence, identification of genetic factors involved in stover quality and subsequent transfer of these factors to elite pearl millet breeding lines lacking the stover quality trait will provide a foundation to improve the stover quality of elite hybrid parental lines and ultimately elite hybrid cultivars themselves.

To improve stover quality, marker-aided selection is expected to provide a powerful tool. Conventional breeding for quantitative traits is often an extremely slow and laborious process and because of genotype x environment interactions, the application of results from such breeding efforts tends to be location specific. The application of DNA markers and mapping technology facilitates breeding for complex traits. After mapping QTL for stover quality in the segregating progeny derived from the cross of a superior stover quality donor parent and an elite parent, markers linked to the

QTL can be employed to transfer these QTL from the donor parent to a recipient parent (recurrent parent). This is particularly easy if the recipient parent is the elite parent of the mapping population used to originally identify the QTL.

There are many types of DNA markers currently available for linkage mapping, QTL identification and marker assisted-breeding. Restriction Fragment Length Polymorphism (RFLP) markers identified by Southern hybridization were the first generation of reliable DNA-based markers. A second generation markers using Polymerase Chain Reaction (PCR) include, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Sequence Characterized Amplified Regions (SCAR), Single Nucleotide Polymorphism (SNP) markers and their many variants. Among them, the simple sequence repeats (SSR) markers, also called as microsatellites are widely used in applied plant breeding programs. SSR markers offer a potentially attractive combination of features that makes them useful as molecular markers in breeding programs. RFLP markers also have the same features except that they require large quantities of DNA and the process is slow, complex, and more expensive. A large number of SSR loci have been genetically mapped in several agronomically important species, including rice (Cho *et al.*, 1997), soyabean (Cregan *et al.*, 1999), sorghum (Taramino *et al.*, 1997; Bhattramakki *et al.*, 2000) and pearl millet (Qi *et al.*, 2004). In most cases these SSR markers have been used to supplement linkage maps originally developed using RFLP markers, such as that originally reported for pearl millet by Liu *et al.* (1994).

In pearl millet, using RFLP and SSR-based genetic linkage map of a moderate-sized mapping population based on the cross ICMB 841 × 863B, ICRISAT and ILRI Scientists have attempted to map QTLs associated with grain and stover yield and aspects of drought tolerance (Hash *et al.*, 2003). Some ruminant livestock feed quality-related attributes of leaf blade, leaf sheath and stem internode stover fractions corresponding to the penultimate leaf of the main stem were assessed in pearl millet stover produced from testcross hybrids of the (ICMB 841 × 863B)-based pearl millet mapping population, and a QTL associated with the digestibility of the leaf blade fraction was detected on linkage group 7. We aimed to tranfer this putative stover quality QTL to elite breeding line, producing pairs of near-isogenic lines for this QTL, in order to better assess the potential utility of this QTL prior to its possible further transfer into the genetic backgrounds of other elite hybrid parental lines. The specific objectives of the present study are:

- to select among backcross progenies ($BC_5F_1/BC_4F_2$ pairs) for introgression of the linkage group 7 stover quality QTL from donor 863B into the genetic background of ICMB 841 based on linked RFLP and SSR markers.

- to minimize the linkage drag accompanying introgression of this QTL (the 863B allele is loosely linked to a QTL for reduced stover yield) by background selection for ICMB 841 marker genotypes in adjacent non-target regions of pearl millet linkage group 7 and on other linkage groups in which full recovery of the recurrent parent alleles has not been completed.

# CHAPTER # 2

# REVIEW OF LITERATURE

## CHAPTER II

## REVIEW OF LITERATURE

Livestock rearing can provide a pathway out of poverty through improvements to household nutrition, cash income, asset building and employment (McIntire *et al.*, 1992; Sansoucy *et al.*, 1995; de Haan *et al.*, 1999). It is highly probable that by exploiting the opportunities represented by the increasing demand for livestock products in developing countries (Delgado *et al.*, 1999), that even higher benefits can accrue for poverty reduction from livestock in years to come. In many parts of the developing world, and especially in South Asia, West Africa and the East African Highlands, livestock productivity is closely linked to the quantity and quality of available fodder, much of which is sourced from food crops. These seasonally available fodders are generally inadequate in quantity and quality, particularly in rain-fed systems and during extended dry seasons, presenting a serious constraint to livestock productivity (Renard, 1997). Most of the livestock keepers are resource poor crop-livestock farmers with very limited access to arable land and water conditions that limit allocation of arable land exclusively for the purpose of fodder production (Renard, 1997). Declining and deteriorating common property resources further reduce access to fodder aggravating the nutritional deficit and reducing livestock productivity.

The pressure from the increased human population, intensifying cropping systems and greater crop-livestock integration has translated into farmers increasingly requiring crops that provide not only good grain or pod yields but also more reliable and better

quality fodder. Whether in South Asia (Kelly *et al.*, 1996, Underwood *et al.*, 2000, Rama Devi *et al.*, 2000), West Africa (Singh *et al.*, 2003) or East Africa (Desta *et al.*, 2000; Romney *et al.*, 2003), the demand expressed by crop-livestock farmers for improved food-feed crops is well documented. By combining the gains from selection for grain, pod and forage components, food-feed crops can contribute to improved resource use and systems efficiencies that will help alleviate fodder constraints without additional demands on scarce agricultural resources such as arable land and water, since these are in any case required inputs for grain and pod production. Furthermore, the delivery of food-feed crop technologies to farmer's field can be greatly aided by short deliver pathways since the private and public players in the seed industry are developing at an encouraging rate in South Asia (Govila *et al.*, 1997; Hall and Yogand, 2000) and elsewhere.

As a consequence, the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) and the International Livestock Research Institute (ILRI) have implemented collaborative research on the genetic improvement of fodder value of food-feed-crops, with emphasis on sorghum and pearl millet, which are important crops for the rural poor. The research has demonstrated the existence of significant genetic variation for fodder quality traits and it has identified cultivars that provide superior stover quality and quantity without detriment to grain yield.

**2.1 Importance of pearl millet stover**

Pearl millet is the most drought tolerant of all domesticated cereals. It can yield under seasonal rainfall as low as 200-250mm (Bidinger and Hash, 2004) making it the only reliably productive cereal in the driest rain-fed regions of the arid and semi-arid

tropics. Nearly 70 per cent of Indian pearl millet area (>9 million hectares) is sown to hybrid seed with most of this purchased by farmers with land holdings of less than five hectares (Govila *et al.*, 1997; Talukdar *et al.*, 1999). In addition to pearl millet's role as major source of calories in human food in areas in which it is widely grown, its stover constitutes a major component of ruminant rations in marginal production environments, particularly during the dry season when green fodder/grazing resources are limited. The nutritional quality of millet stover is relatively poor however, and supplies are often inadequate, both of which are linked to the low productivity of livestock in smallholder crop-livestock systems in these environments (Renard, 1997)

Practices that might improve stover yields and stover quality, such as higher applications of nitrogen (currently averaging only 5-20 kg/ha, as both manure and mineral fertilizer) are considered very risky for farmers to adopt in such highly unpredictable, drought-prone environments. The best option for increasing the availability and quality of crop residues appears to be genetic improvement of both these characteristics in currently available cultivars. Such cultivars, which have characteristically been bred only for a high grain yield, are considered by farmers to have an inferior stover quality, in comparison to adapted, but lower yielding, traditional landraces (Kelly *et al.*, 1996). Considering the growing demand for more and better quality fodder for livestock, pearl millet improvement programs need to become multidimensional, targeting the whole plant rather than one single trait.

**2.2 Genetic variation for stover quality**

Wide variation for stover quality parameters has been documented in few studies. Blummel *et al.* (2003) investigated the ruminant nutritional quality of six prominent cultivars of pearl millet grown under low (9 kg/ha) and high (90 kg/ha) nitrogen fertilizer, in similar feeding trials with bulls to those of sorghum. Stover organic matter digestibility in bulls varied among genotypes from 40.1 to 48.1 per cent and from 45.3 to 51.3 per cent under high and low fertilizer application, respectively. Daily digestible organic matter intake also differed. Despite these differences among cultivars, no cultivar had sufficient stover quality (at either fertilizer level) when fed without supplementation, to meet animal maintenance requirements, as all estimated changes in live weight of bulls were negative. In addition, estimated changes in live weight in bulls and grain yield genotypes tended to be inversely associated ($P<0.1$), suggesting that simultaneous improvement of both grain yields and animal performance may be difficult to achieve. These data also showed that fertilizer application did not necessarily improve pearl millet stover quality.

These initial conclusions were re-evaluated in a recent, more comprehensive study involving a greater number (30) of more genetically variable genotypes of pearl millet (divided equally among arid zone landraces, improved open-pollinated varieties and high yielding hybrids), which were grown at three very different locations in India (Gwalior, Nagaur and Patancheru). Genotypes differed significantly for biomass, grain and stover yields, and for stover nutritional quality characteristics. The range in genotype means for stover digestibility was significant (39 to 42%), but smaller than that in the

initial experiment, but the range in digestible stover yields was large (900 to 1800 kg/ha), due to a large range in stover yield. Digestible dry matter intake (estimated by a combination of Near Infrared Reflectance Spectroscopy (NIRS), laboratory traits and *in vivo* experimentation with sheep) ranged from 25 to 32 g/kg $LW^{0.75}$/d, which was both larger than that in the initial study and overall significantly higher with sheep than bulls. Heritability for all stover quality and yield traits were of same order as that for grain yield (0.6 to 0.7), which was sufficiently high to the expected useful progress from selection for improved stover quality traits.

In summary, this study indicated that there were useful and heritable genetic variation in important stover quality traits in pearl millet, and a high degree of independence between stover fodder value and grain yield over a wide range of genotypes and evaluation environments confirming that there is an opportunity to improve both traits in breeding programs. With nearly 70 per cent of the Indian pearl millet area sown to modern hybrids, the improvement in the quantity and/or nutritional quality of the stover of these hybrids could make a significant impact on livestock productivity in millet growing areas. In response to this opportunity, ICRISAT and ILRI have initiated research on dual purpose top-cross hybrids, generated from both grain and dual purpose pollinators and fodder-type male sterile lines, to provide private hybrid seed industry with improved parental lines that convey higher stover quality without sacrificing grain and stover yield (Blummel and Rai, 2004).

## 2.3 Molecular breeding of pearl millet

### 2.3.1 RFLP markers and Linkage maps in pearl millet

In recent times, the use of molecular marker technology for the genetic improvement of pearl millet has made some headway, and pearl millet has been elevated to the status of a molecular crop thanks to a series of collaborative projects involving John Innes Centre (JIC) and ICRISAT supported by the Plant Sciences Research Programme of the UK's Department for International Development (Breese *et al.*, 2002). The first genetic map of pearl millet was generated by Liu *et al.* (1994). The map contained 181 Restriction Fragment Length Polymorphism (RFLP) marker loci and spanned a genetic distance of 303 cM. Currently, the map contains 242 loci and spans 473 cM. The difference in length is mainly due to the addition of 12 distally located markers. A subset of these markers has subsequently been transferred to a series of different crosses that segregate for agronomically important traits.

Recently, genetic linkage maps of four different pearl millet crosses have been integrated to develop a consensus map of about 353 RFLP (220 homologous and 133 heterologous RFLP markers) and 65 SSR markers (Qi *et al.*, 2004). An interesting feature of the genetic maps of pearl millet is the extreme localization of recombination towards the chromosome ends. The concentration of mapped markers in centromeric regions, reflecting an unequal distribution of recombination, was first observed in the early molecular maps of wheat (Chao *et al.*, 1989) and has since been seen in several species (Devos *et al.*, 1992; Qi *et al.*, 1996; Tanksley *et al.*, 1992), but it appears to be extreme in pearl millet. Physical mapping of one such region on linkage group 1 revealed a physical

mapping to genetic distance ratio of <12 kb/cM (F.K. Padi and K.M. Devos, unpublished). This unequal distribution of recombination appears to be largely cross-independent, and will have consequences for the transfer of traits from donor to elite pearl millet germplasm.

The integration of markers previously mapped in other grass species has provided the anchor points to align the pearl millet linkage groups to other cereal genetic maps, including the cereal model, rice. Although the pearl millet genome appears to be relatively highly rearranged relative to rice, regions of colinearity between the two species can be clearly identified (Devos *et al.*, 2000). These now form a framework for exploitation of the rice genomic sequence as a source of new markers and candidate genes underlying traits in pearl millet.

**2.3.2 Microsatellite markers for pearl millet**

The genetic tools for marker-assisted breeding of pearl millet are now in place and available for anyone to use in improving pearl millet hybrids and to extend the economic lifespan of elite hybrid parental lines. However, application of these discoveries is hampered by the limited availability of repeatable, polymorphic PCR-compatible markers in pearl millet. Although ICRISAT and its partners have successfully demonstrated the use of RFLP markers in the transfer of downy mildew resistance and enhanced terminal drought tolerance, these markers are too labor-intensive and high cost for applied use, as well as having potential health and environmental hazards. Thus RFLP

markers are not considered suitable for large-scale genotyping applications in an applied plant breeding program.

For plant breeding applications, PCR-compatible markers based on microsatellites or simple sequence repeats (SSRs) are often considered the most appropriate. SSRs typically provide single-locus markers, which are often co-dominantly inherited and characterized by hypervariability, abundance and reproducibility. However, development of SSR markers is expensive as it requires a substantial investment in DNA sequencing. To date, circa 100 SSR markers are available for use in pearl millet (Qi *et al.*, 2001; Allouis *et al.*, 2001; Budak *et al.*, 2003; Qi *et al.*, personal communication), but a much larger number is required for their application in plant breeding. Therefore, development of additional SSR markers is a valuable objective for the pearl millet research community.

In the past, SSRs have been expensive to develop and this has largely limited their application to the more commercially important crops. Enrichment protocols have been used to reduce these costs by focusing sequencing efforts on DNA clones that are likely to contain a particular repeat motif (*e.g.*, Budak *et al.*, 2003). Recently however, an alternative source of microsatellites has been utilized. Discovery of microsatellites in Expressed Sequence Tags (ESTs) provides the opportunity to develop SSR markers in a simple and direct way, *i.e.*, by electronic searches (data mining) of EST databases. Exploitation of this source of SSR markers is obviously limited to the species for which EST sequence information is available. This specific approach was first attempted in rice

(Miyao *et al.*, 1996) and has subsequently been reported from many other crops including pearl millet (Senthilvel *et al.*, 2004).

**2.3.3 QTL mapping for stover quality:**

QTL mapping has become an important tool in understanding responses to many traits contributed or controlled by many genes. It provides a means to dissect complex phenotypic characters such as drought tolerance, yield, stover quality, height and allows the identification of molecular markers linked to desirable QTLs, so that these can be directly used in Marker-Assisted Selection (MAS). QTL for disease resistance (Jones *et al.*, 1995, 2002), drought tolerance (Yadav *et al.*, 2002, 2004), flowering time and grain and stover yield (Yadav *et al.*, 2003) and characters involved in domestication (Poncet *et al.*, 2000, 2002) have been mapped in pearl millet.

An attempt has also been made to identify QTL associated with stover yield and quality parameters in pearl millet in a mapping population involving the cross between ICMB 841B and 863B (Hash *et al.*, 2003). The linkage map was constructed based RFLP and SSR genotypic data on 160 $F_2$ individuals. Linkage groups were named according to common anchor markers with the map of Liu *et al.* (1994). The genetic map length and distribution of markers for this population was comparable to the consensus map of pearl millet (Devos *et al.*, 2000) and to other maps published for this species (Jones *et al.*, 1995; Yadav *et al.*, 2002). Using this map, the stover yield QTLs were identified on linkage group 2, 5 and 7 and were unaffected by Genotype × Environment interaction. Only one genomic region associated with stover yield (mapping on LG6) was

significantly affected by G×E interaction. Genomic regions associated with stover yield also co-mapped with the regions associated with grain yield and harvest index (Yadav *et al.*, 2002) but parental alleles associated with increased effects on these traits having reducing effects on stover yield. This further clarified that these genomic regions contributed to increased grain yield by their effects on increased partitioning of assimilates from stover to the filling grains under stress conditions.

QTL mapping of stover yield and quality parameters revealed several stover yield QTL, but none appeared to be independent of flowering time and/or plant height QTL that are normally expected to be associated with stover, grain and biomass yield. However, it has proven difficult to detect statistically significant QTL for in vitro measurements associated with ruminant nutritional quality. Some reasons for this include the relatively low operational heritability of the *in vitro* measurements (due to part of the *in vitro* measurements have been conducted on only a portion of the available field replicates) and due to the small numbers of mapping population progenies (79) used in the field trial.

The best putative QTL initially detected for *in vitro* measures, has been for gas production from the leaf blade fraction of the stover, which maps to the top of LG7 of 863B, and accounts for about 20 per cent of the observed variation for this trait in mapping population progeny hybrids produced with only one of the two testers. In order to identify more QTL for stover quality traits, it will probably be necessary to move over to NIRS-based predictions of quality component traits from scans of ground stover

samples (in order to assess all samples from all field replicates), and to use larger numbers of mapping population progenies in order to allow detection of statistically significant QTL that account for more modest portions of the observed phenotypic variation. This increase in effective mapping population size will probably require that we use only one tester.

**2.3.4 Marker assisted breeding**

Selection of a genotype carrying desirable gene or gene combination via linked marker(s) is called marker-aided selection (MAS). Breeders practice marker aided selection when an important trait, that is difficult to assess, is tightly linked to another Mendelian trait, which can be easily scored. Most traits of agronomic importance, including yield, nutritional quality and stress tolerance, are quantitatively inherited (Allard, 1960; Hallauer and Miranda, 1988). The ability to manipulate genes responsible for quantitative traits is a prerequisite for sustained improvement of crop plants. In this context, MAS has been advocated as a useful tool for rapid genetic advance in case of QTL (Lande and Thompson, 1990; Knapp, 1994, 1998). Gimelfarb and Lande (1995) presented detailed analysis of the relationship between genetic markers and QTL in the process of MAS.

Mohan *et al.* (1997) suggested that MAS could be used to pyramid major genes including disease and insect resistance genes, with the ultimate goal of producing the crop cultivars with more desirable traits. A study conducted by Eathington *et al.* (1997) assessed the usefulness of marker-assisted effects estimated from early generation

testcross data for predicting later generations testcross performance. Witcombe and Hash (2000) have described how multiple resistance gene pyramids can be used practically to strategically deploy resistance genes.

MAS appears particularly promising when dominant alleles at QTL are present and linked in coupling phase when compared to phenotypic selection (Van Berloo and Stam, 1998). The advent of molecular marker techniques has had a large impact on quantitative genetics. Marker based methods applied to segregating population have provided with a means to locate QTL to chromosomal regions and to estimate the effects of QTL allele substitution (Lander and Botstein, 1989). In backcross breeding program, it has been shown that MAS can be effective in reducing linkage drag and optimizing population sizes, by selecting against the donor genome except for alleles to be introduced from donor.

Hospital *et al.* (1997) used the computer simulations to study the efficiency of MAS based on an index combining the phenotypic value and molecular score of individuals. They observed that in the first generation the ratio of relative efficiency (RE) of expected efficiency of MAS over the expected efficiency of purely phenotypic selection generally increases when considering 1) the larger population size 2) lower heritability values of the trait and 3) high type-I error risk of the regression. These studies over the successful generations of the rate of fixation of QTL shows that the higher efficiency of MAS on QTL with large effects in early generation is balanced by a higher rate of fixation of unfavorable alleles at QTL with small effects in later generations. This

explains why MAS may become less efficient than phenotypic selection in long-term process. MAS efficiency therefore depends on the genetic determination of that trait.

The efficiency of MAS was generally reduced with increasing the distance between the markers. So, optimal distance recommended between two adjacent markers is about 5-10cM. The efficiency of marker-assisted selection is less efficient than the phenotypic selection in long-term process (Hospital *et al.*, 1997).

Knapp (1998) presented the estimates of probability of selecting one or more superior genotypes by MAS to estimate its cost efficiency relative to phenotypic selection. The frequency of superior genotypes among the selected progeny increases as the selection intensity increases. Charmet *et al.* (1999) studied the accuracy of QTL location determination greatly affects selection efficiency. MAS for QTL have started to be applied to the genetic improvement of quantitative character in several crops such as tomato (Lowson *et al.*, 1997; Bernacchi *et al.*, 1998), maize (Graham *et al.*, 1997), and barley (Han *et al.*, 1997; Toojinda *et al.*, 1998).

Hospital and Charcosset (1997) determined the optimal position and number of marker loci for manipulating QTL in foreground. Further, they investigated the combination of foreground and background selection in QTL introgression. Openshaw (1994) determined the population size and marker density required in background selection. Frisch *et al.* (1999) determined the number of marker data points (MDP) required in background selection, size of the population and compared a two-stage

selection procedure (one background and one fore ground selection), with alternative selection procedures (one foreground and two or three background selection steps). They concluded that as the number of selection processes increases, the number of MDP required decreases.

Moreau *et al.* (2000) evaluated the relative efficiency of MAS in the first cycle of selection through an analytical approach taking into account the effect of experimental design (population size, number of trials and replication/trial) on QTL detection. They concluded that expected economic returns of MAS compared to the phenotypic selection decreases with the cost of genotyping.

With this background, ICRISAT has initiated the marker-assisted introgression of a pearl millet stover quality QTL using the RFLP and SSR markers. For ruminant nutritional quality of stover fractions, the marker-assisted backcross transfer of the putative leaf blade gas QTL from LG7 of 863B (Hash *et al.*, 2003) to ICMB 841 is in progress and it is now advanced to $BC_4F_2$ and $BC_3F_1$ progenies. Each generation progenies were analysed using SSRs and RFLP markers flanking the QTL to find whether the gene has been introgressed or not.

# CHAPTER #3

# MATERIALS AND METHODS

## CHAPTER III

## MATERIALS AND METHODS

### 3.1 Plant material

A set of 20 pearl millet (*Pennisetum glaucum*) $BC_4F_2$ and $BC_5F_1$ families (consisting of about 25 plants each) derived from crosses between donor parent 863B and recurrent parent ICMB 841 were analysed for marker-assisted introgression of a putative stover quality QTL. The schematic diagram of the backcrossing is given in Figure 1.

Donor parent 863B was bred at ICRISAT-Patancheru by direct selfing and selection within a sample of *Iniadi* landrace material from Togo. It has large grain size (and associated broader leaf blades, thicker stems, and thicker panicles), drought tolerance, downy mildew resistance, and stover quality. It was originally selected based on its combination of agronomic preference and superior combining ability for terminal drought tolerance.

The recurrent parent ICMB 841 is also an agronomically superior seed parent maintainer line genotype with good combining ability for grain and stover yield and it is the seed parent maintainer line of several popular dual-purpose hybrids released in India. Compared to 863B, ICMB 841 has smaller seed size (and associated narrower leaf blades, thinner panicles, and thinner stems), and has poorer combining ability for terminal drought tolerance, but has similar plant height and flowering time.



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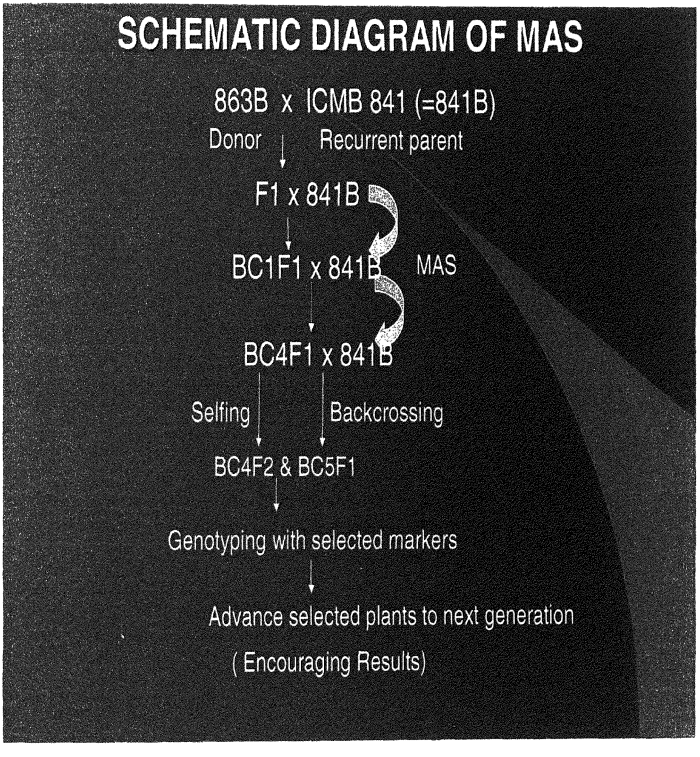
**Figure 1. Scheme for transfer of stover quality QTL from donor parent 863B to elite pearl millet seed parent maintainer line ICMB 841 by marker-assisted selection and recurrent backcrossing**



Some ruminant livestock feed quality-related attributes of leaf blade, leaf sheath and stem internode stover fractions corresponding to the penultimate leaf of the main stem were assessed in pearl millet stover produced from testcross hybrids of the (ICMB 841 × 863B)-based pearl millet mapping population, and a QTL associated with the digestibility of the leaf blade fraction was detected on linkage group 7 (Hash *et al.*, 2003) for which drought tolerance QTLs were reported recently by Yadav *et al.* (2004).

### 3.2 DNA extraction

Seeds of backcross progeny from selected individuals and the parents were sown in the field. Staggered sowing was employed to ensure co-flowering of the recurrent parent and backcross progenies. DNA from the $BC_4F_2$ and $BC_5F_1$ populations was extracted from about 15 days old seedlings as per the following protocols.

#### 3.2.1 Small scale DNA extraction

Small quality of DNA from a large number of samples was extracted following the protocol described by Mace *et al* (2003). The steps involved are described below.

**Preparation**

- Steel balls (2 per extraction tube), pre-chilled at –20°C for about 30 minutes, were added to the extraction tubes which were kept on ice.
- 3% CTAB buffer (3% w/v CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl, pH 8.0, 0.17% β-mercaptoethonol) was pre-heated in 65°C water bath before start of sample collection.

- Six inches long leaf strips were collected (final weight 30–40mg) from one-week-old seedlings cut in to pieces (1mm in length). These cut leaf were transferred to the extraction tubes.

**Grinding and extraction**

- 450µl of pre-heated 3% CTAB buffer was added to each extraction tube containing leaf sample.
- Grinding was carried out using Sigma GenoGrinder at 500 strokes/minute for 5 minutes.
- Grinding was repeated until the color of solution becomes pale green and cut leaf was sufficiently macerated.
- After grinding, the tube box was fixed in a locking device and incubated at +65°C in a water bath for 10 minutes with occasional manual shaking.

**Solvent extraction**

- 450µl of chloroform – iso-amylalcohol (C:IAA=24:1) mixture was added to each tube and the samples were centrifuged at 6200 rpm for 10 minutes.
- After centrifugation the aqueous layer was transferred to a fresh tube (approximately 300µl)

**Initial DNA precipitation**

- To each tube containing aqueous layer, 0.7 volume (approximately 210µl) of cold (kept at –20°C) isopropanol was added, then solution was carefully mixed and the tubes were kept at –20°C for 10 minutes.
- The samples were centrifuged at 6200 rpm for 15 minutes.

- The supernatant was decanted under and pellets were allowed to air dry (minimum 20 minutes).

**RNase treatment**

- In order to remove RNA 200µl of low salt TE buffer and 30mg of RNase (stock 10 mg/µl) were added to the each tube containing dry pellet and mixed properly.
- The solution was incubated at 37°C for 30 minutes.

**Solvent extraction**

- After incubation, 200µl of phenol – chlorofom – iso-amylalcohol (IAA) mixture (25:24:1) was added to each tube carefully mixed and centrifuged at 5000 rpm for 10 minutes.
- The aqueous layer was transferred to the fresh tubes and the step was repeated with the chloroform – IAA mixture.

**DNA precipitation**

- To the tubes containing aqueous layer 15µl (approximately $1/10^{th}$ volume) 3M Sodium acetate and 300µl (2 vol) 100% ethanol was added and subsequently placed in freezer for 5 minutes.
- Following incubation, the box with tubes was centrifuged at 6200 rpm for 15 minutes.

**Ethanol wash**

- After centrifugation, supernatant was carefully decanted and to the pellets 200µl of 70% ethanol was added followed by centrifugation at 5000 rpm for 5 minutes.

**Final re-suspension**

- Pellets obtained by carefully decanting the supernatant and allowed to air dry for one hour.
- Completely dried pellets were resuspended in 100µl of $T_{10}E_1$ buffer and kept at room temperature to dissolve completely.
- Dissolved DNA samples were kept in +4°C.

By this method, we could extract DNA from 96 samples at time in a single day. However, the DNA could be used only for SSR analysis since the concentration was too less to be used in RFLP analysis. For RFLP analysis DNA was extracted from 5g leaf samples.

### 3.2.2 Large scale DNA Extraction

**Extraction**

- 5 g of plant sample (young pearl millet leaves) was weighed on a piece of aluminium foil and dipped in liquid nitrogen.
- The frozen sample was transferred to the pre-cooled mortar and ground into a fine powder.
- Powder was transferred into a 50 ml plastic conical tube containing 15–20 ml of 'S' buffer (pre-heated), mixed gently and incubated in 65°C water bath for about 45 minutes.

- 50µl of proteinase K was added to the tubes and incubated in water bath at 65°C for 40 minutes (tubes mixed at intervals of 5 minutes)
- Equal volume of equilibrated phenol was added and centrifuged at 5000 rpm for 15 minutes in a refrigerated centrifuge.
- The top aqueous layer was transferred into a new tube. To that, 20 ml of phenol – chloroform (1:1) was added and centrifuged at 5000 rpm for 15 minutes.
- The top aqueous layer was removed and an equal volume of chloroform – iso-amyl alcohol (24:1) was added and centrifuged.
- The top aqueous layer was removed and transferred to a new tube and an equal volume of cold isopropanol was added .
- With the help of a glass hook, the DNA was spooled out and transferred into a 15 ml polypropylene tube and washed three times with 1ml of 70% ethanol and air-dried.
- The DNA pellet was dissolved in 2.5–3 ml of $T_{50}E_{10}$ depending on the size.
- 30µl of RNase (10 mg/ml) was added to the DNA solution and incubated at 4°C or at room temperature overnight.

**Purification**

- After overnight incubation, the RNase treated samples were incubated at 37°C for 30 minutes.
- Equal volume of phenol (2.5–3 ml) was added, mixed and centrifuged at 5000 rpm for 5 minutes.

- The supernatant was collected into another glass tube and equal volume of phenol – chloroform (1:1) was added and centrifuged at 5000 rpm for 5 minutes.
- The top aqueous layer was removed and an equal volume of chloroform (chloroform – iso-amyl alcohol, 24:1) was added to that. It was centrifuged for 5 minutes at 5000 rpm.
- The aqueous layer was collected in a new tube added with $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) and an equal volume of cold isopropanol.
- The DNA was spooled out with a glass hook into a 1.5 ml eppendorf tube and washed with 1ml of 70% ethanol.
- The DNA pellet was resuspended in $T_{10}E_1$ (400–500µl) depending on the size of the pellet.

### 3.2.3 DNA quality and quantity check

The DNA quality was checked using 1% agarose gels. For checking the quality, 1µl of DNA was mixed with 1µl of orange dye and 8µl of distilled water and loaded onto 1% agarose gel. The gel was run for 10 minutes, after which the quality was checked under UV. A DNA smear indicates poor quality whereas an intact and clear band indicates good quality DNA. Samples of poor quality were re-extracted. The quantity of DNA was tested using spectrophotometer.

**3.3 Genotyping**

SSR markers linked to the putative QTL for pearl millet stover quality were used for foreground selection to identify the individuals presumed to have the 863B donor parent allele in the target region (foreground selection). Donor parent alleles for the foreground markers indicate the presence of the stover quality QTL allele from the donor parent. However, the tighter the markers are linked to the QTL, the greater the chance that the QTL in between both markers has been transferred. However, in the end phenotypic testing of the final products of the marker assisted breeding, exercise will need to be performed in order to confirm the transfer of this stover quality QTL. At the same time, recurrent parent alleles for the markers unlinked to the target stover quality QTL have been used to select those individuals with minimal linkage drag (background selection). The RFLP markers flanking the stover quality QTL were used in foreground selection to ascertain the allele present at the putative stover quality QTL to allow selection of the individuals probably having the donor parent alleles in this genomic region. RFLP markers were not used for background selection.

The genetic linkage map of pearl millet cross ICMB 841 × 863B and the position of stover quality QTL is shown in Figure 2. The SSR and RFLP markers selected for genotype screening are given in Table 5.

**Figure 2. Genetic Linkage map of pearl millet cross ICMB 841 X 863B**

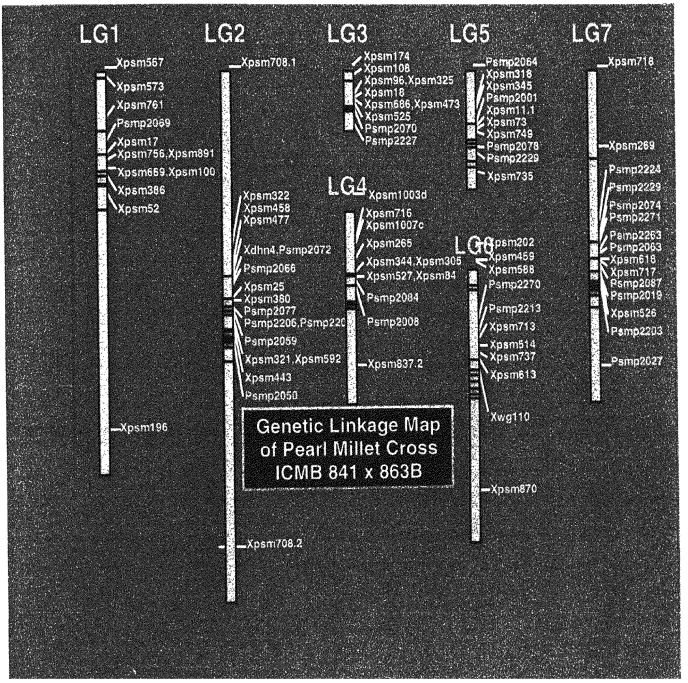


**Table 5: The SSR and RFLP markers selected for genotype screening.**

| Generation | Number of Individuals Screened | Linkage group/ Targeted QTL | Markers screened |
|---|---|---|---|
| BC4F2 | 148 | LG-1 | *Xpsmp* 2273 |
| | | LG-2 | *Xpsmp* 2201, *Xpsmp* 2231, *Xpsmp* 2225, *Xpsmp* 2059 |
| | | LG-3 | *Xpsmp* 2267 |
| | | LG-4 | *Xpsmp* 2076, *Xpsmp* 2084 |
| | | LG-5 | *Xpsmp* 2202 |
| | | LG-6 | *Xpsmp* 2270, *Xpsmp* 2213 |
| | | LG-7 | *Xpsmp* 2013, *Xpsmp* 2040, *Xpsmp* 2043, *Xpsmp* 2019 *Xpsmp* 2027 |
| BC5F1 | 17 (individuals of family bulked) | LG-1 to LG-7 | Same as listed above |
| BC4F2 | 21 | LG-7/Stover Quality QTL | *Xpsm* 718, *Xpsm*269, *Xpsm* 618, *Xpsm* 526 |
| BC5F1 | 93 | LG-7/Stover Quality QTL | *Xpsm* 718, *Xpsm* 269, *Xpsm* 618, *Xpsm* 526 |

**Note: *Xpsmp* are SSR markers/primers and *Xpsm* are RFLP probes**

### 3.3.1 SSR analysis

#### 3.3.1.1 PCR amplification

PCR was performed in 96-well plates using the SSR primer pairs (Table 1) in a Perkin Elmer (Norwalk Conn.) DNA thermocycler. The reactions were performed in volumes 10µl and the PCR reaction mixture contains 10mM Tris-HCl (pH 8.3), 50mM KCl, 10ng of DNA, 2 pmol of forward and reverse primers, 1mM $MgCl_2$, 0.2mM of each dNTP and 0.5U of *Taq* DNA polymerase. The PCR program consisted of initial denaturation for 5 minutes at 94°C and then denaturation for 45 seconds at 94°C, annealing at 58 to 61°C for 45 seconds, and extension at 72°C for 45 seconds with 35 cycles. The last PCR cycle was followed by a final extension of 5 minutes at 72°C to ensure amplification to equal length of both DNA strands.

#### 3.3.1.2 Electrophoresis

The PCR products were separated on 6% non-denaturing Polyacrylamide Gel Electrophoresis (PAGE) and the products were resolved using silver staining procedure. To the PCR product, 2µl of loading dye (Orange red + EDTA + NaCl + Glycerol) was added. From these mixture, 2µl of sample was loaded onto the 6% non-denaturing PAGE. The gel was prepared using 52.5ml of doubled distilled water, 7.5ml of 10X TBE buffer, 15ml of acrylamide: bis-acrylamide (29:1) solution, 450µl of ammonium persulphate and 100µl of TEMED. Along with the samples, 100bp marker (50ng / µl) was also loaded in the first and last lane of the gel to ensure proper sizing of amplified

PCR fragments. The gel was run at 600–650 V of constant power in 0.5X TBE buffer for 3 hours using a Bio-Rad sequencing gel apparatus.

After running of PAGE gels for required time, the gels were silver stained and the sequential steps involved in silver staining were:

The gel was placed in the following solutions with continuous shaking

- water for 5 minutes
- 0.1% CTAB solution for 20 minutes (2 g in 2l of water)
- 0.3% ammonia solution for 15 minutes (26ml of 25% ammonia in 2l of water)
- 0.1% silver nitrate solution for 15 minutes (2g of silver nitrate + 8ml of 1M NaOH in 2l of water and neutralize with ammonia solution till the solution becomes colorless)
- Developer (30g of sodium carbonate + 400 µl of formaldehyde in 2l of water) till clear products were visible

After developing of the bands, gels were rinsed in water for 1 minute and placed in fixer (30ml glycerol in 2l of water) for few minutes.

After silver staining of the PAGE gels, the size (base pairs) of the intensely amplified specific bands or alleles for each SSR marker was estimated based on their migration relative to the 100 bp DNA ladder (fragments ranging from 100 bp to 1000 bp) and presence or absence of parental alleles were scored.

### 3.3.2 RFLP Analysis

#### 3.3.2.1 Digestion of genomic DNA:

Around 23 μg of genomic DNA was digested using appropriate restriction enzymes and the digestion reaction was set up according to the following protocol:

| | |
|---|---|
| DNA (1μg/μl) | - 23 μl |
| 10X buffer | - 3 μl |
| Bovine Serum Albumin | - 3 μl |
| Restriction enzyme (25 units) | - 0.6 μl |

Sterile distilled water was added to make up the total reaction volume to 30 μl and Mixed by pulse centrifugation was incubated at 37°C for overnight.

#### 3.3.2.2 Electrophoresis of digested genomic DNA and Southern blotting

The restriction enzyme digested DNA samples were separated on 1% agarsose gel using 1X TAE buffer for 4-5 hours at 100V and the following steps were followed for southern transfer of digested DNA into a nylon membrane.

- Ethidium bromide was removed as much possible by soaking the gel in sterile distilled water (SDW)
- Then the gel was depurinated by soaking the gel in 0.25 N HCl for 15 minutes to allow more efficient transfer of larger DNA fragments.
- The blotting sheets were cut to the size of the gel.
- Hybond N+ nylon membrane and 3–4 sheets of Whatman 3MM filter paper were also cut to the size of the agarose gel

- A bridge in a glass tray was made using a bit tough sponges. Wicks were made with 2–3 sheets of Whatman 3MM filter paper and soaked in 0.4 N NaOH.
- Glass tray was filled with 0.4 N NaOH and the agarose gel was placed on top of Whatman 3MM filter paper bridge.
- Then nylon membrane was carefully placed on the top of the gel in an inverted position so that fragments are near to the nylon membrane.
- Thin glass rod or pipette was rolled to remove the air bubbles between the gel and the membrane.
- Two Whatman 3MM filter paper sheets cut to size were placed on the membrane after soaking in 0.4 N NaOH.
- A stack of blotting paper sheets which were cut to size of the gel were placed on top of Whatman 3MM filter paper.
- After placing the blotting sheets, it was ensured that there was no direct contact between blotting sheets and 0.4 NaOH except through the gel.
- A glass plate of appropriate size on top of the blotting paper stack was placed and a weight of 0.5 kg was placed on the glass plate to make the transfer uniform across the gel.
- The setup was left overnight and the stack of blotting sheets were removed the next day
- Nylon membrane was carefully removed with the help of forceps.
- Then it was washed in 2X SSC for 15 minutes. Dried and wrapped in Saranwrap® and kept in cold till use.

- The complete transfer was ensured by soaking the gel in ethidium bromide and viewing under UV light.

**3.3.2.3 Southern Hybridization:**

Before the filters/blots can be probed with specific nucleic acid molecules, it is necessary to pre-treat them with carrier DNA that effectively blocks any naked DNA binding sites exposed on the membrane surfaces. This prevents non-specific binding of the probe on the entire blot. The following steps were followed for Hybridization of specific probes to the sample DNA:

**Pre-hybridization**

- 1 ml of salmon sperm DNA (ssDNA) was taken in a 1.5 ml eppendorf and kept at 90°C for 5 minutes for denaturation
- Place the heat denatured salmon sperm DNA on ice immediately.
- The blots were placed in hybridization bottles and 30–40 ml of distilled water was added.
- The bottles were kept in oven at 65°C for rotation for 5 minutes and after 5 minutes distilled water was removed and 20–30 ml of prehybridisation solution was added.
- 1 ml of salmon DNA solution (denatured) was added and kept in hybridization oven for 3 hours at 65°C if the blot was an old blot and 6 hours if it was a new blot.

**Radioactive labeling of Probes**

- 5 µl of DNA fragment to be used as probe was taken in an eppendorf tube.
- 20 µl of sterile distilled water (SDW), 0.5 µl of *Hind*III-digested λ DNA (control DNA) was added, mixed and spinned.
- The probe along with SDW and control DNA was incubated at +90°C for 5 minutes to denature and immediately chilled on ice for 5 minutes.
- Then the following reagents are added,

| | |
|---|---|
| 10X labeling buffer | - 5 µl |
| dNTPs | - 6 µl |
| Klenow enzyme | - 1 µl |
| [α- $^{32}$P ] dATP | - 2 µl |

- Mixed and spinned before incubating.
- And incubated at 37°C for more than an hour.

**Hybridization**

- 5µl of 0.2M EDTA (pH 8.0) and 145µl SDW was added to the incubated sample.
- Then the contents were mixed, spinned and kept at 90°C for 5 minutes and got down to room temperature immediately by placing on ice.
- Half volume of pre-hybridization solution was taken out and the contents containing the radioactive substance was added to the bottles containing the blots.

- The hybridizing bottles were then placed in the hybridization oven for over-night rotation at 65°C (~16 hrs).

**Washing**

- After overnight rotation of the blots in the hybridization oven at 65°C, the radioactive solution was removed from the hybridizing bottles.
- The blots were washed with 65–80 ml of solution I containing 20X SSC and 20% SDS, 100ml and 25ml respectively, (make up the volume to 1L)
- The blots were kept in hybridization oven at +65°C for 15 minutes to remove the excess hybridization buffer. The blots were washed with solution I twice.
- Solution I was discarded and 65-80 ml of solution II (10ml 20X SSC, 25ml 20% SDS, and make up the volume to 1L) was added.
- The blots were Kept in oven for 15 minutes for rotation and solution II was discarded. Then the blots were washed with distilled water finally.
- The blots were collected carefully and laid on top of a filter paper to dry or can be dried by keeping them in hybridization oven.

**Autoradiography**

- After drying the radioactivity was tested by the monitor. The blot was marked for orientation. Then the blot was exposed to X-ray film cassette with high intensifying screens.
- The cassettes containing the blots were kept at –80°C for varying periods depending on the radioactive counts present on the blots as determined by Geiger Muller (GM) counter.

**Stripping**

- The process of removing the radioactive probes from the blots is called stripping. The blots are washed with the stripping solution about 3–4 times to remove the labeled probe. Stripping solution contains 25ml of 20X SSC, and 5ml of 20% SDS in 1L of water.

**3.4 Data collection and selection of genotypes**

The bands on the gels were scored for the presence of parental alleles. The individuals showing banding pattern of donor parental type (863B) was scored as 'A' and recurrent parental type (ICMB 841) was scored as 'B'. The heterozygotes having both parental alleles were scored as 'H'.

SSRs analysis was done using a set of 16 primers in both background and foreground selection and RFLP analysis was performed using 4 enzyme-probe combinations, which were linked with the putative stover quality QTL to identify the introgression of the target region in the backcross progenies. $BC_5F_1$ progenies segregating 1:1 for heterozygosity and $BC_4F_2$ segregating1:2:1 for marker alleles in the vicinity of the putative leaf blade gas QTL from donor 863B were selected based on SSR and RFLP marker genotype data.

The progenies were also tested for the presence of ICMB 841 SSR marker alleles for the non-target region of linkage group 7 and a set of SSR markers from other linkage

groups. The selected plants were selfed / backcrossed and advanced to next generation, in which individuals having homozygous 863B genotype in the target region will be identified and multiplied (by selfing) prior to being evaluated for their stover quality parameters.

# CHAPTER #4

# RESULTS

# CHAPTER IV

# RESULTS

## 4.1 Quality and quantity of DNA

The quality of the DNA extracted by both small and large scale extraction methods was good. The intactness of genomic DNA after agarose gel electrophoresis is shown in Plate 1 and Plate 2. The $OD_{260nm}$ / $OD_{280nm}$ measured by spectrophotometer was in the range of 1.52 to 1.8 indicating the purity of the DNA. The DNA concentration was in the range of 300 to 400ng/µl (Plate 1) in small scale extraction whereas it was in the range of 1.3 to 3.4µg/µl (Plate 2) in Maxi-prep extraction. All DNA samples were normalized to a final concentration of 5ng/µl for SSR analysis and 1µg/µl for RFLP analysis.

## 4.2 SSR genotyping

A set of 16 polymorphic SSR markers were screened on 165 progenies of both $BC_4F_2$ (148 individuals) and $BC_5F_1$ (17 bulks) to detect the segregating populations in these progenies by running the PCR amplified products on 6% PAGE gels. Progenies were selected for SSR analysis based on uniformity for the phenotypic characters of the recurrent parent like a profusely hairy leaf blade, which is governed by the recessive gene *hl*, which maps to pearl millet linkage group 6 (C.T. Hash, unpublished).

Eleven SSR markers representing LG 1 to 6 were used for background selection. It was found that all the progenies were homozygous for the alleles from the recurrent



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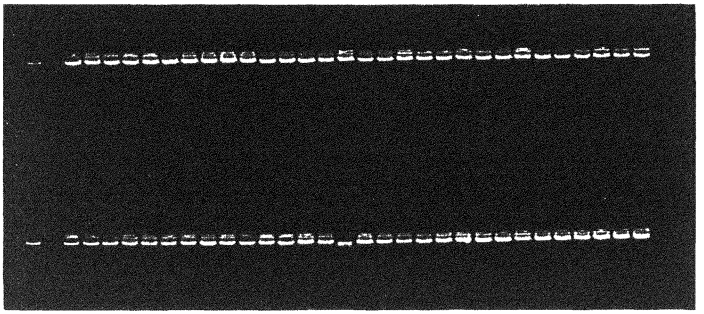
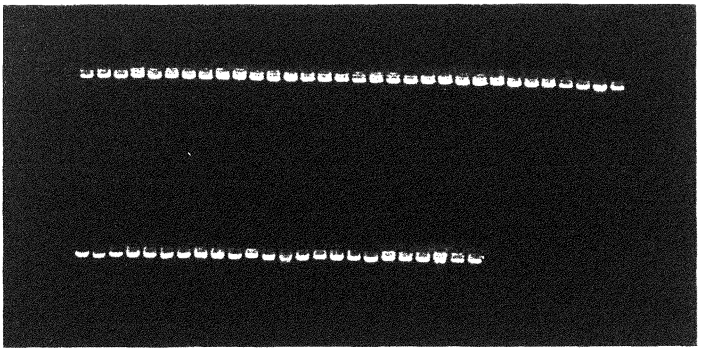
**Plate 1: Intactness of DNA and concentration of HTP extracted (small scale) DNA (300-400ng/µl)**



**Note:** The number of genotypes included in the above plate are only 60 out of 165 genotypes selected for SSRs genotyping.

**Plate 2: Concentration of large scale extracted DNA (maxi-prep)**



**Note:** The number of genotypes included in the above plate are only 56 out of 114 selected for maxi-prep DNA extraction.

parent at most of these loci, and hence scored "B", as expected based on marker data generated in earlier generations of the backcrossing program (Satish Kumar, unpublished). This recovery of recurrent parent background in regions unlinked with the target region is subjected to foreground selection. In this case, background marker genotyping was performed primarily to confirm that theoretical expectations were being met and that no inadvertent outcrossing had occurred in the most recent generation of selfing and backcrossing [Table 1. (a) & (b)]. Except for linkage group 2 (LG2), [Table 2. (a)], the recurrent parent alleles were fully recovered in all of the progenies evaluated. However, parts of LG2 seem to still be segregating with 2 SSR markers and with the other 2 markers on LG2, the recurrent parent alleles have been fully recovered [Table 2. (b)]. Based on this data, the individuals homozygous for the recurrent parent allele (i.e., those scored "B") are selected and RFLP analysis was done for those genotypes.

Foreground selection was also done using primers for 5 SSR marker loci mapping to pearl millet LG7. These 5 markers showed the segregating patterns in the ratio of 1:1 (A:H) for the $BC_5F_1$ populations and 1:2:1 (A:H:B) for the $BC_4F_2$ populations. It clearly indicated that PCR amplification using SSR primer pairs, followed by silver staining of PAGE-separated amplification products provides an effective way to screen the segregating populations and select the progenies for further analysis. Amplification for the primers *Xpsmp* 2043 and 2040 are shown in Plate 3 and 4 and genotyping data of SSR primers Xpsmp 2027 and Xpsmp 2019 are given in Table 3. (a) & (b). Based upon the SSR data generated, 114 out of the 165 progenies were selected for further analysis.

**Table 1: Background selection by SSRs markers**

**(a) Scoring chart for the primer *Xpsmp* 2273 (LG-1)**

| Family | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P (17) | B | B | B | B | B | B | B | B | B | B | B | B | B | - | B | B | B |
| 328 (11) | B | B | B | B | B | B | B | B | - | B | B | -- | | | | | |
| 330 (20) | B | B | B | B | B | B | B | B | B | B | B | B | - | B | B | B | B |
| | B | B | B | -- | | | | | | | | | | | | | |
| 332 (32) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | B | B | B | B | B | B | B | B | B | B | B | B | B | - | -- | |
| 338 (34) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | - | B | B | B | B | B | B | B | - | B | B | - | B | B | B | B |
| 340 (18) | B | B | B | - | B | B | B | B | B | B | B | B | B | B | - | B | B |
| | B | -- | | | | | | | | | | | | | | | |
| 342 (33) | B | - | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | - | B | B | B | B | B | B | - | B | B | B | B | B | B | B | B | -- |

**(b) Scoring chart for the primer *Xpsmp* 2084 (LG-4)**

| Family | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P (17) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| 328 (11) | B | B | - | B | B | B | B | B | B | B | B | -- | | | | | |
| 330 (20) | B | B | B | B | B | B | B | B | B | B | B | B | - | B | B | B | B |
| | B | B | B | -- | | | | | | | | | | | | | |
| 332 (32) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | B | B | B | B | B | B | B | B | B | B | B | B | B | - | -- | |
| 338 (34) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | - | B | B | B | B | B | B | B | - | B | B | - | B | B | B | B |
| 340 (18) | B | B | B | - | B | B | B | B | B | B | B | B | B | B | - | B | B |
| | B | -- | | | | | | | | | | | | | | | |
| 342 (33) | B | - | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | - | B | B | B | B | B | B | - | B | B | B | B | B | B | B | B | -- |

* Xpsmp are SSR Primers and LG represents Linkage Group
* 'B' scored for 841B allele (Recurrent Parent)

**Table 2: Background selection by SSRs markers**

**(a) Scoring chart for the primer *Xpsmp* 2201 (LG-2)**

| Family | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P (17) | H | B | H | B | B | B | H | H | H | H | H | H | B | B | B | H | H |
| 328 (11) | B | B | H | H | H | H | B | B | - | A | H | -- | | | | | |
| 330 (20) | B | H | H | A | H | H | A | H | B | B | A | B | H | A | A | H | A |
| | B | H | B | -- | | | | | | | | | | | | | |
| 332 (32) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | B | B | B | B | B | B | B | B | B | B | B | - | B | B | -- | |
| 338 (34) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | - | B | B | B | B | B | - | B | B | B | B | B | B | - | B | B | B |
| 340 (18) | B | B | B | A | A | A | A | A | A | A | - | A | A | A | - | - | A |
| | A | -- | | | | | | | | | | | | | | | |
| 342 (33) | - | - | - | A | - | A | - | B | - | H | H | A | H | A | B | - | B |
| | - | - | A | - | B | - | B | A | B | B | H | B | B | - | B | - | -- |

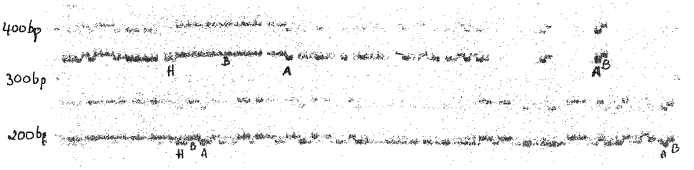
**(b) Scoring chart for the primer *Xpsmp* 2231 (LG-2)**

| Family | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P (17) | B | B | B | B | B | B | B | B | - | B | B | B | B | B | B | - | B |
| 328 (11) | B | B | B | B | - | - | B | B | - | - | B | -- | | | | | |
| 330 (20) | B | - | B | A | B | B | - | B | B | B | A | B | B | B | B | B | B |
| | B | - | B | -- | | | | | | | | | | | | | |
| 332 (32) | B | - | B | B | - | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | B | B | B | B | B | B | B | - | B | B | B | B | - | B | -- | |
| 338 (34) | B | B | B | - | B | B | B | - | - | B | B | B | B | - | B | B | A |
| | A | B | B | A | B | B | B | B | A | A | B | B | B | A | A | B | A |
| 340 (18) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | -- | | | | | | | | | | | | | | | |
| 342 (33) | B | - | B | B | B | B | B | B | B | B | B | B | B | - | B | B | B |
| | - | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | -- |

* Xpsmp are SSR Primers and LG represents Linkage Group

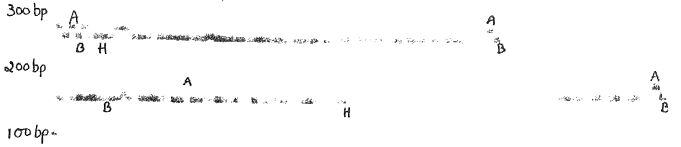
* 'B' scored for 841B allele, 'A' for 863B allele and 'H' for Heterozygote

**Plate 3: PAGE gel for SSR marker *Xpsmp* 2043 for all BC4F2 & BC5F1 progenies (a total of 165 genotypes)**




* *Xpsmp* 2043 = 192/200 bp
bp = base pair
'A' allele - 863B (Donor Parent)
'B' allele - 841B (Recurrent Parent)
'H' allele - Heterozygote

**Plate 4: PAGE gel for the SSR primer *Xpsmp* 2040 for all BC4F2 & BC5F1 progenies (165 genotypes)**




* *Xpsmp* 2040 = 163 bp
bp = base pair
'A' allele - 863B (Donor Parent)
'B' allele - 841B (Recurrent Parent)
'H' allele - Heterozygote

**Table 3: Foreground selection by SSR markers**

**(a) Scoring chart for the primer *Xpsmp* 2027 (LG-7)**

| Family | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P (17) | B | B | H | H | H | H | - | B | B | B | H | H | H | H | B | - | H |
| 328 (11) | B | H | H | B | B | - | B | B | H | H | A | -- | | | | | |
| 330 (20) | B | - | H | B | B | B | B | A | B | B | H | B | H | H | B | H | H |
| | A | B | H | -- | | | | | | | | | | | | | |
| 332 (32) | H | H | H | B | H | H | H | H | A | H | H | B | H | A | A | H | H |
| | B | H | B | H | H | B | B | H | - | H | H | B | - | H | H | -- | |
| 338 (34) | H | H | B | H | H | B | B | A? | A? | A? | A? | B | H? | H? | H? | H? | B |
| | A | H | B | A | B | H | B | A | H | B | B | A | H | A | H | B | B |
| 340 (18) | A | A | H | H | H | B | B | H | H | H | H | H | H | B | H | H | A |
| | H | -- | | | | | | | | | | | | | | | |
| 342 (33) | H | - | H | H | H | H | H | H | H | B | H | H | H | H | A | H | A |
| | - | H | H | H | B | B | B | - | H | H | H | H | H | A | A | H | -- |

**(b) Scoring chart for the primer *Xpsmp* 2019 (LG-7)**

| Family | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P (17) | B | B | H | H | B | B | B | B | H | B | H | B | B | B | B | H | B |
| 328 (11) | H | H | B | B | B | A | H | B | H | H | H | -- | | | | | |
| 330 (20) | B | B | H | B | B | B | H | H | B | B | A | H | B | - | - | H | H |
| | H | B | A | -- | | | | | | | | | | | | | |
| 332 (32) | H | H | H | H | H | H | H | H | A | H | A | H | - | A | H | H | H |
| | H | H | B | B | H | B | B | H | H | A | H | H | H | A | A | -- | |
| 338 (34) | H | B | B | B | H | H | H | H | H | A | B | H | H | B | A | A | H |
| | H | A | B | A | B | B | B | A | A | A | A | A | A | A? | A? | H? | H? |
| 340 (18) | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B |
| | B | -- | | | | | | | | | | | | | | | |
| 342 (33) | A | - | H | H | H | A | A | H | H | A | H | H | A | H | H | H | H |
| | - | B | A | H | H | B | H | H | B | A | H | H | A | B | A | B | -- |

* Xpsmp are SSR primers and LG represents the Linkage Group.
'A' scored for 863B (donor allele), 'B' for 841B (recurrent allele) and 'H' for heterozygote.

**4.3. RFLP genotyping**

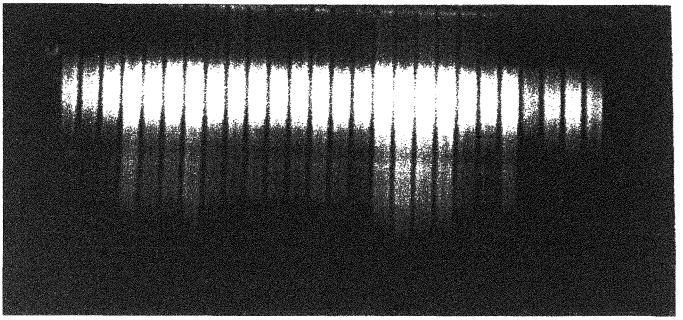
Ninety-three individuals from 17 $BC_5F_1$ families (the plants of each family were bulked for SSR analysis) and 21 individuals from $BC_4F_2$ populations were selected for further RFLP analysis as RFLP loci *Xpsm*718 and *Xpsm*269 flank the putative QTL target region and there were no polymorphic SSR loci available that had previously been mapped to this portion of pearl millet LG7.

Genomic DNA extracted from the individuals selected based on SSR marker data was first digested with restriction enzymes *Hind*III, *Dra*I, *Eco*RI and *Eco*RV for Southern blotting and hybridization. Smears were obtained when the digested samples were subjected to agarose gel electrophoresis in TAE buffer, indicating proper digestion of DNA samples by each of the restriction enzymes [Plate 5. (a), (b), (c) & (d)].

After Southern blotting of the gels by the capillary method, Hybond N+ membrane blots containing the DNA fragments were labelled with PCR-amplified probes PgPSM718, PgPSM269, PgPSM618, and PgPSM526, which when probed against electrophoretically-separated digests of particular restriction enzymes detect loci mapping to pearl millet LG7. Autoradiography of the blots revealed that the $BC_5F_1$ individuals are segregating in a 1:1 ratio for the heterozygous condition (scored "H") and homozygosity for the recurrent parent ICMB 841 allele (scored "B") [Plate 6]. The subset of $BC_4F_2$ individuals that were subjected to RFLP analysis were observed to be segregating for the heterozygous condition (scored "H") and homozygosity for the donor parent 863B allele (scored "A").

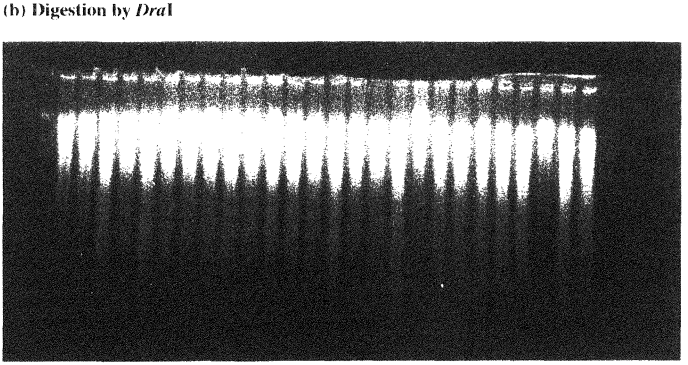
**Plate 5: Digestion of Genomic DNA by Restriction Enzymes.**

**(a) Digestion by *Hind*III**



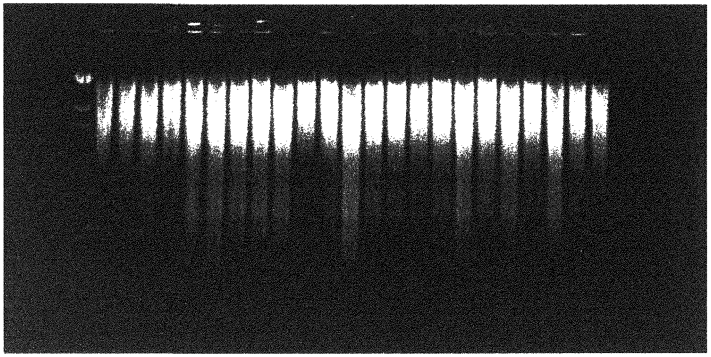
**Note:** The number of DNA samples in the above plate are only 24 among 114 samples digested by the Restriction Enzyme *Hind*III. Parents 841B & 863B are also digested.
Marker (λ *Hind*III digest) is loaded in the first well, then 2 parents and 24 genotypes.

**(b) Digestion by *Dra*I**



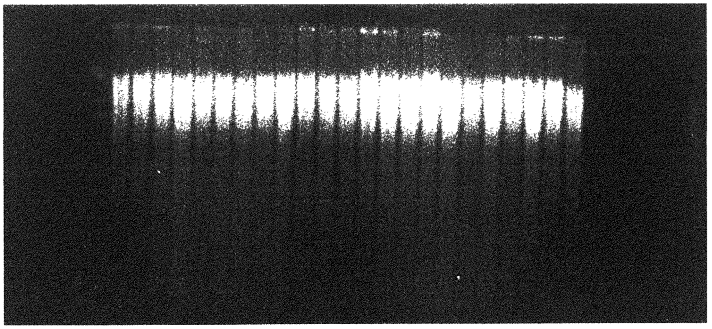
**Note:** The number of DNA samples in the above plate are only 24 among 114 samples digested by the Restriction Enzyme *Dra*I. Parents 841B & 863B are also digested.
Marker (λ *Hind*III digest) is loaded in the first well, then 2 parents and 24 genotypes.

**(c) Digestion by *EcoR*I**



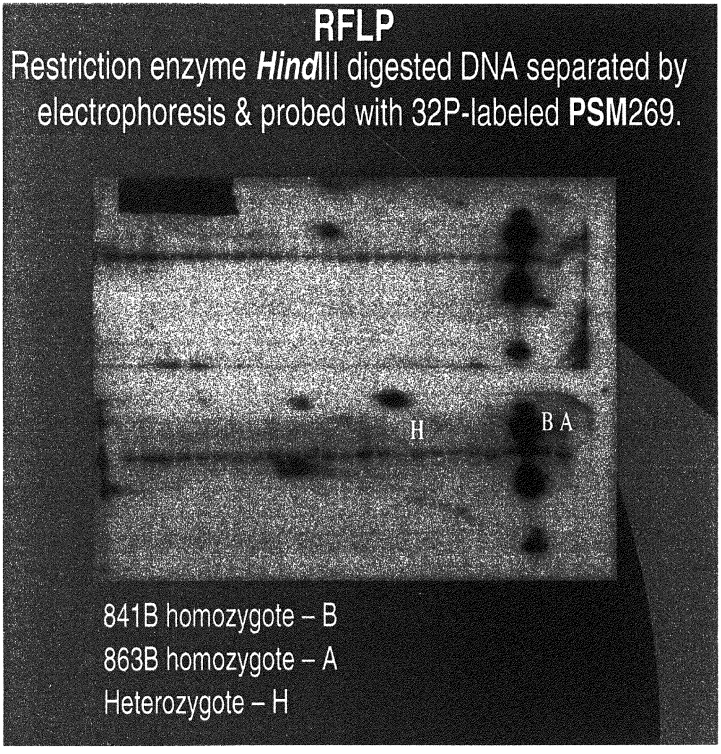
**Note:** The number of DNA samples in the above plate are only 21 among 114 samples digested by the Restriction Enzyme *EcoR*I. Parents 841B & 863B are also digested.
Marker (λ *Hind*III digest) is loaded in the first well, then 2 parents and 21 genotypes

**(d) Digestion by *EcoR*V**



**Note:** The number of DNA samples in the above plate are only 21 among 114 samples digested by the Restriction Enzyme *EcoR*V. Parents 841B & 863B are also digested.
Marker (λ *Hind*III digest) is loaded in the first well, then 2 parents and 21 genotypes

**Plate 6: Autoradiograph of HindIII/PSM 269 enzyme-probe combination**



**4.4 Marker-assisted Selection of backcross progenies**

Based on both RFLP and SSR data sets [Table 4], it was observed that the markers linked to stover quality QTL present on the seventh linkage group are still segregating in the $BC_4F_2$ and $BC_5F_1$ populations. The individuals scored "H" or "A" for loci on LG7 (foreground selection) and "B" for loci on all other linkage groups (background selection) were selected and have been advanced to the next generation by selfing ($BC_4F_2$ and $BC_5F_1$ individuals) and/or backcrossing ($BC_5F_1$ individuals only), and their derivatives will be evaluated for stover quality parameters by conducting *in vivo* and *in vitro* feeding trails on ruminant livestock.

Table 5: The SSR and RFLP markers selected for genotype screening of generation BC4F2 and BC5F1.

**Table 4: Selected SSR genotype data and RFLP data on LG-7 for both BC4F2 and BC5F1 progenies (114 genotypes)**

ble 4: Selected SSR genotype data and RFLP data on LG-7 for both BC4F2 and BC5F1 progenies (114 genotypes)

| SR No. | S.No. | Pl.No. | 2273 | 2201 | 2225 | 2231 | 2059 | 2267 | 2076 | 2084 | 2202 | 2270 | 2213 | 718 | 269 | 2013 | 2027 | 618 | 2043 | 2019 | 526 | 2040 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 1 | 313_1 | B | B | B | B | B | B | B | B | B | _ | B | | B | H | C | B | B | B | B | C |
| | 2 | 313_2 | | | | | | | | | | | | | | | | | | | | |
| 6 | 3 | 314_1 | B | B | H | B | H | B | B | B | B | B | B | | B | H | C | B | B | B | B | B |
| | 4 | 314_2 | | | | | | | | | | | | | | | | | | | | |
| | 5 | 314-5 | | | | | | | | | | | | | | | | | | | | |
| | 6 | 314_6 | | | | | | | | | | | | | | | | | | | | |
| | 7 | 314_7 | | | | | | | | | | | | | | | | | | | | |
| | 8 | 314_8 | | | | | | | | | | | | | | | | | | | | |
| 7 | 9 | 315_1 | B | H | H | B | H | B | B | B | B | H | B | | B | H | _ | B | B | B | B | B |
| | 10 | 315_3 | | | | | | | | | | | | | | | | | | | | |
| | 11 | 315_4 | | | | | | | | | | | | | | | | | | | | |
| | 12 | 315_5 | | | | | | | | | | | | | | | | | | | | |
| 8 | 13 | 316_1 | B | H | B | B | H | B | B | B | B | B | _ | | H | H | B | B | B | B | B | B |
| | 14 | 316_2 | | | | | | | | | | | | | | | | | | | | |
| | 15 | 316_3 | | | | | | | | | | | | | | | | | | | | |
| | 16 | 316_4 | | | | | | | | | | | | | | | | | | | | |
| | 17 | 316_5 | | | | | | | | | | | | | | | | | | | | |
| 10 | 18 | 318_1 | B | H | B | B | H | B | B | B | B | H | H | | H | _ | B | | B | B | B | B |
| | 19 | 318_2 | | | | | | | | | | | | | | | | | | | | |
| | 20 | 318_3 | | | | | | | | | | | | | | | | | | | | |
| | 21 | 318_4 | | | | | | | | | | | | | | | | | | | | |
| | 22 | 318_5 | | | | | | | | | | | | | | | | | | | | |
| | 23 | 318_6 | | | | | | | | | | | | | | | | | | | | |
| 12 | 30 | 320_1 | B | H | B | B | H | B | B | B | B | H | H | | B | H | H | H | H | B | B | B |
| | 31 | 320_2 | | | | | | | | | | | | | | | | | | | | |
| | 32 | 320_3 | | | | | | | | | | | | | | | | | | | | |
| | 33 | 320_4 | | | | | | | | | | | | | | | | | | | | |
| | 34 | 320_6 | | | | | | | | | | | | | | | | | | | | |
| | 35 | 320_7 | | | | | | | | | | | | | | | | | | | | |
| 13 | 36 | 321_1 | B | B | B | B | B | B | B | B | B | B | B | | H | H | H | B | B | B | B | B |
| | 37 | 321_2 | | | | | | | | | | | | | | | | | | | | |
| | 38 | 321_3 | | | | | | | | | | | | | | | | | | | | |
| | 39 | 321_4 | | | | | | | | | | | | | | | | | | | | |
| | 40 | 321_5 | | | | | | | | | | | | | | | | | | | | |
| | 41 | 321_6 | | | | | | | | | | | | | | | | | | | | |
| | 42 | 321_7 | | | | | | | | | | | | | | | | | | | | |
| | 43 | 321_8 | | | | | | | | | | | | | | | | | | | | |
| | 44 | 321_9 | | | | | | | | | | | | | | | | | | | | |
| | 45 | 321_10 | | | | | | | | | | | | | | | | | | | | |
| | 46 | 321_11 | | | | | | | | | | | | | | | | | | | | |
| | 47 | 321_12 | | | | | | | | | | | | | | | | | | | | |
| | 48 | 321_13 | | | | | | | | | | | | | | | | | | | | |
| | 49 | 321_14 | | | | | | | | | | | | | | | | | | | | |
| | 50 | 321_15 | | | | | | | | | | | | | | | | | | | | |
| | 51 | 321_16 | | | | | | | | | | | | | | | | | | | | |

| SR No. | S.No. | Pt.No. | [redacted] | | | | | [redacted] | | | [redacted] | | | [redacted] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2273 | 2201 | 2225 | 2231 | 2059 | 2267 | 2076 | 2084 | 2202 | 2270 | 2213 | 718 | 269 | 2013 | 2027 | 618 | 2043 | 2019 | 526 | 2040 |
| 14 | 52 | 322_1 | _ | B | B | B | B | B | B | B | B | B | B | | B | H | H | H | H | B | B | B |
| | 53 | 322_2 | | | | | | | | | | | | | | | | | | | | |
| | 54 | 322_3 | | | | | | | | | | | | | | | | | | | | |
| | 55 | 322_4 | | | | | | | | | | | | | | | | | | | | |
| | 56 | 322_5 | | | | | | | | | | | | | | | | | | | | |
| | 57 | 322_6 | | | | | | | | | | | | | | | | | | | | |
| | 58 | 322_7 | | | | | | | | | | | | | | | | | | | | |
| | 59 | 322_8 | | | | | | | | | | | | | | | | | | | | |
| | 60 | 322_9 | | | | | | | | | | | | | | | | | | | | |
| | 61 | 322_10 | | | | | | | | | | | | | | | | | | | | |
| | 62 | 322_11 | | | | | | | | | | | | | | | | | | | | |
| | 63 | 322_12 | | | | | | | | | | | | | | | | | | | | |
| | 64 | 322_13 | | | | | | | | | | | | | | | | | | | | |
| | 65 | 322_14 | | | | | | | | | | | | | | | | | | | | |
| | 66 | 322_15 | | | | | | | | | | | | | | | | | | | | |
| | 67 | 322_16 | | | | | | | | | | | | | | | | | | | | |
| | 68 | 322_17 | | | | | | | | | | | | | | | | | | | | |
| 15 | 69 | 323_1 | B | B | H? | B | B | B | B | B | B | H | B | | B | H | B | B | B | B | B | B |
| | 70 | 323_2 | | | | | | | | | | | | | | | | | | | | |
| | 71 | 323_3 | | | | | | | | | | | | | | | | | | | | |
| | 72 | 323_4 | | | | | | | | | | | | | | | | | | | | |
| | 73 | 323_5 | | | | | | | | | | | | | | | | | | | | |
| | 74 | 323_6 | | | | | | | | | | | | | | | | | | | | |
| | 75 | 323_7 | | | | | | | | | | | | | | | | | | | | |
| 16 | 76 | 324_1 | B | H | B | _ | B | B | B | B | B | B | _ | | H | H | _ | H | H | H | B | B |
| | 77 | 324_2 | | | | | | | | | | | | | | | | | | | | |
| | 78 | 324_3 | | | | | | | | | | | | | | | | | | | | |
| | 79 | 324_4 | | | | | | | | | | | | | | | | | | | | |
| | 80 | 324_6 | | | | | | | | | | | | | | | | | | | | |
| | 81 | 324_7 | | | | | | | | | | | | | | | | | | | | |
| | 82 | 324_8 | | | | | | | | | | | | | | | | | | | | |
| | 83 | 324_9 | | | | | | | | | | | | | | | | | | | | |
| | 84 | 324_10 | | | | | | | | | | | | | | | | | | | | |
| | 85 | 324_11 | | | | | | | | | | | | | | | | | | | | |
| | 86 | 324_12 | | | | | | | | | | | | | | | | | | | | |
| | 87 | 324_14 | | | | | | | | | | | | | | | | | | | | |
| | 88 | 324_15 | | | | | | | | | | | | | | | | | | | | |
| | 89 | 324_16 | | | | | | | | | | | | | | | | | | | | |
| 17 | 90 | 325_1 | B | H | B | B | B | B | B | B | B | B | B | | B | H | H | B | B | B | B | B |
| | 91 | 325_2 | | | | | | | | | | | | | | | | | | | | |
| | 92 | 325_3 | | | | | | | | | | | | | | | | | | | | |
| | 93 | 325_4 | | | | | | | | | | | | | | | | | | | | |
| 11 | 24 | 319_1 | B | H | H? | B | H | B | B | B | B | H | H | | B | H | H | H | H | H | H | B |
| | 25 | 319_2 | | | | | | | | | | | | | | | | | | | | |
| | 26 | 319_3 | | | | | | | | | | | | | | | | | | | | |
| | 27 | 319_5 | | | | | | | | | | | | | | | | | | | | |
| | 28 | 319_6 | | | | | | | | | | | | | | | | | | | | |
| | 29 | 319_7 | | | | | | | | | | | | | | | | | | | | |

| ISR No. | S.No. | Pl.No. | 2273 | 2201 | 2225 | 2231 | 2059 | 2267 | 2076 | 2084 | 2202 | 2270 | 2213 | 718 | 269 | 2013 | 2027 | 618 | 2043 | 2019 | 526 | 2040 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 21 | 94 | 328_4 | B | H | B | B | H | B | B | B | B | B | H | | H | B | B | B | B | B | B | A |
| 25 | 95 | 328_8 | B | B | B | B | B | B | B | B | B | B | B | | H | H | B | B | B | B | B | B |
| 29 | 96 | 330_1 | B | B | B | B | B | B | B | B | B | H | B | | H | H | B | B | B | B | B | B |
| 32 | 97 | 330_4 | B | A | B | A | A | B | B | B | B | H? | B | | B | H | B | H | B | B | B | B |
| 43 | 98 | 330_15 | B | A | B | B | B | B | B | B | B | B | B | | B | H | B | B | B | _ | B | B |
| 61 | 99 | 332_13 | B | B | H | B | B | B | B | B | B | B | B | | A | H | H | H | H | _ | H | H |
| 62 | 100 | 332_14 | B | B | A | B | B | B | B | B | B | B | B | | A | _ | A | H | A | A | A | H |
| 83 | 101 | 338_3 | B | B | B | B | B | B | B | B | B | H | B | | _ | B | B | B | B | B | B | B |
| 100 | 102 | 338_20 | B | B | B | B | B | B | B | B | B | B | B | | A | _ | B | H | B | B | B | B |
| 101 | 103 | 338_21 | B | B | B | A | B | B | B | B | B | B | B | | A | _ | A | H | A | A | B | A |
| 102 | 104 | 338_22 | B | B | B | B | B | B | B | B | B | B | B | | H | _ | B | B | B | B | B | B |
| 104 | 105 | 338_24 | B | _ | B | B | B | B | B | B | B | B | B | | H | _ | B | B | B | B | B | B |
| 105 | 106 | 338_25 | B | B | B | B | B | B | B | B | B | B | B | | A | _ | A | H | A | A | B | B |
| 115 | 107 | 340_1 | B | B | B | B | B | B | B | B | B | H | B | | B | _ | A | B | B | B | B | B |
| 116 | 108 | 340_2 | B | B | B | B | B | B | B | B | B | H | B | | B | _ | A | B | B | B | B | B |
| 120 | 109 | 340_6 | B | A | B | B | B | B | B | B | B | B | B | | _ | _ | B | B | B | B | B | B |
| 128 | 110 | 340_14 | B | A | B | B | B | B | B | B | B | B | B | | B | _ | B | B | B | B | B | B |
| 131 | 111 | 340_17 | B | A | _ | B | B | B | B | B | B | B | B | | B | _ | A | B | B | B | B | B |
| 142 | 112 | 342_19 | B | H | B | B | B | B | B | B | B | _ | B | | H | _ | B | H | A | A | B | B |
| 147 | 113 | 342_15 | B | B | B | B | B | B | B | B | B | _ | B | | A? | _ | A | H | H | H | B | _ |
| 149 | 114 | 342_17 | B | B | B | B | B | B | B | B | B | H | B | | B | _ | A | H | H | H | B | H |

**Note:** Probe Xpsm 718 was not scored as it was not polymorphic.

841B Homozygote – 'B' allele

863B Homozygote – 'A' allele

Heterozygote - 'H' allele

**Table 5: The SSR and RFLP markers selected for genotype screening.**

| Generation | Number of Individuals Screened | Linkage group/ Targeted QTL | Markers screened |
|---|---|---|---|
| BC4F2 | 148 | LG-1 | *Xpsmp* 2273 |
| | | LG-2 | *Xpsmp* 2201, *Xpsmp* 2231, *Xpsmp* 2225, *Xpsmp* 2059 |
| | | LG-3 | *Xpsmp* 2267 |
| | | LG-4 | *Xpsmp* 2076, *Xpsmp* 2084 |
| | | LG-5 | *Xpsmp* 2202 |
| | | LG-6 | *Xpsmp* 2270, *Xpsmp* 2213 |
| | | LG-7 | *Xpsmp* 2013, *Xpsmp* 2040, *Xpsmp* 2043, *Xpsmp* 2019, *Xpsmp* 2027 |
| BC5F1 | 17 (individuals of family bulked) | LG-1 to LG-7 | Same as listed above |
| BC4F2 | 21 | LG-7/Stover Quality QTL | *Xpsm* 718, *Xpsm*269, *Xpsm* 618, *Xpsm* 526 |
| BC5F1 | 93 | LG-7/Stover Quality QTL | *Xpsm* 718, *Xpsm* 269, *Xpsm* 618, *Xpsm* 526 |

**Note: *Xpsmp* are SSR markers/primers and *Xpsm* are RFLP probes**

# CHAPTER #5

# DISCUSSION

# CHAPTER V

# DISCUSSION

Stover constitutes a major component of ruminant rations in marginal production environments, particularly during the dry season when green fodder / grazing are limited. Low productivity of livestock in smallholder crop-livestock systems in these environments is due in part to the limited quantity and low nutritional quality of the available stover (Renard, 1997). The best option for increasing the availability and quality of crop residues appears to be genetic improvement of both these characteristics in locally adapted cultivars.

To date, limited attention has been made by cereal breeders to either the quantity or quality of the stover, despite the high value placed on this component of the crop by farmers in crop-livestock systems. This study attempted to redress this problem by focusing on genetically improving the yield and quality of stover, while maintaining the grain yield and other important agronomic traits of maintainer line (ICMB 841) of well adapted, farmer-accepted hybrids by exploiting the available molecular marker tools in backcross breeding programme.

Molecular markers can increase the efficiency of the breeding process in several ways. Flanking markers can be used to identify the backcross lines that are heterozygous for target genome regions. Advancing only these selected lines will also have the effect of reducing linkage drag (Young and Tanksley, 1989; Tanksley and Nelson, 1996).



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Single-copy or low-copy markers with defined map locations, such as RFLPs and SSRs, are ideal for this step. Molecular markers could also increase the efficiency of backcrossing by allowing for selection of genotypes with maximum percentage of the recurrent parent genome.

With marker-assisted selection (MAS), we could introgress the putative stover quality QTL (associated with leaf blade digestibility) allele from the donor parent 863B to the recurrent parent ICMB 841. At present this marker-assisted backcrossing program is at a stage where it has now been advanced up to the $BC_4F_3$ and $BC_5F_2/BC_6F_1$ seed generations based on the SSR and RFLP genotypic data generated on $BC_4F_2$ / $BC5F_1$ in this study. Depending upon the SSR and RFLP genotyping data of the immediate progenitor progenies of these generations, several of the families will be advanced further. Marker genotype, especially for the RFLP foreground markers, are being taken into consideration during selection of genotypes to be advanced. These markers linked to the putative stover quality gas QTL from the donor 863B were used for foreground selection; that is, the genotypes scored "A" or "H" at these loci on the seventh linkage group are being selected. The markers unlinked to the QTL were used for background selection, (i.e., individuals and progenies scored "B" on all other linkage groups have been selected).

The presence of "B" genotypes for background markers (SSRs) and "A" and/or "H" genotypes for foreground markers (both SSRs and RFLP) ensures the recovery of recurrent parent genome ICMB 841 recombined with the 863B stover quality QTL allele

introgressed. Genotypes meeting the above criteria will be selected, and seed of the next generation (selfed and/or backcrossed) sown for further generation advance. Genotypes scored "B" for all background as well as foreground markers in this next will be selected for use as near-isogenic controls when the selected homozygous QTL introgression lines are evaluated.

The number of markers used for background screening will be decreased in each successive backcross generation because once the recurrent parent allele has been fixed at any given non-target locus, it is not necessary to continue screening at that locus in subsequent generations as the locus will remain homozygous for the rest of the breeding program (Morris et al., 2003).

Once the recurrent parent genome recovery is obtained for all markers outside the target stover quality QTL target region, the heterozygotes in which the QTL introgression is assumed to be present are selfed and/or backcrossed. The resulting $BC_nF_2$ progenies will be genotyped to identify putative stover quality gas QTL introgression homozygotes, that are then multiplied by selfing and testcrossed for field evaluation of agronomic performance including grain and stover yield potential, tolerance to abiotic stresses, and resistance to pests and diseases. Stover harvested from these field trials will then be assessed by NIRS, *in vitro*, and *in vivo* methods for variation in stover quality characters including the ruminant digestibility of the leaf blade stover fraction. After testing, if the stover quality of the QTL introgression line and its hybrids are found significantly better than that of the near-isogenic controls, and at least as good as the original ICMB 841 and

its hybrids for other agronomically important traits, it can be released as a new hybrid parental line itself and/or can be used as an elite donor parent of the stover quality QTL for its introgression into other hybrid parental lines of pearl millet.

The target of this study was to get early access to large straw samples of near-isogenic lines and hybrids for *in vivo* feeding trials that can be used to assess the utility of the two alleles at this putative QTL in terms of direct livestock performance measures like milk production and live weight gains, before applying marker-assisted selection in a large-scale breeding program to introgress the favorable allele into a range of elite pearl millet hybrid parental line backgrounds. Both phenotypic and genotypic characters of any quantitative trait should be taken into consideration for selecting an individual for that quantitative character. In this context, although the putative stover quality QTL is introgressed, further QTLs should be detected or can be identified by moving over to NIRS-based predictions of stover quality component traits from scans of ground stover samples like digestibility, nutritive value, and crude protein content (CPC); and using larger numbers of mapping population progenies in order to allow detection of statistically significant QTLs. With this we can have an elite hybrid parental line having good stover quality and quantity without sacrificing grain yield and quality which is an important criteria of dual-purpose crops grown in crop-livestock production systems.

The fact that QTL mapping and Marker-Assisted Selection (MAS) technology is so challenging that is rapidly emerging as a powerful tool suitable for dealing with target traits that are expensive, difficult or time consuming to evaluate accurately in a plant breeding program. Introgression of these quantitative traits not possible by conventional

breeding practices and strategies is made possible by marker-assisted selection. These tools are the result of the melding together of Mendelian, molecular and quantitative genetics. Technology development, including automation, allele-specific diagnostics, and DNA chips, will make MAS approaches based on large-scale screening much more powerful and effective (Young, 1999).

# CHAPTER #6

# SUMMARY

## CHAPTER VI

## SUMMARY

Pearl millet is the only reliably productive cereal in the driest rainfed parts of South Asia where integrated crop-livestock production systems are the norm. Improving the nutritive value of the straw/fodder and the efficiency of their use in mixed diets is an important option for increasing the livestock productivity of the region.

Identification of genetic factors involved in stover quality which is controlled by quantitative trait loci (QTL) and subsequent transfer of these factors to elite pearl millet breeding lines allows to maximize adoption of improved material and application of DNA markers and mapping technology facilitates breeding for complex traits like stover quality.

The research project focus primarily on use of marker-assisted backcrossing (MAB) to transfer quantitative trait loci (QTLs) for laboratory fodder quality traits shown to be highly predictable of weight gain in sheep fed on pearl millet stover. ICRISAT and ILRI scientists (Hash *et al.*) in collaboration have attempted to map this QTL associated with grain and stover yield and aspects of drought tolerance in pearl millet based on RFLP and SSR marker linkage map obtained from the mapping population of the cross ICMB 841 X 863B. QTL mapping of stover yield and quality parameters in pearl millet has revealed several stover yield QTLs (Hash *et al., 2003*) and the best putative stover



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quality QTL detected to date is for gas production from the *in vitro* digestion of ground stover samples.

Each generation progenies in the marker assisted introgression of stover quality QTL (i.e.) BC4F2 and BC5F1, about 165 genotypes were analysed using polymorphic SSR markers and 114 individuals were analysed using RFLP markers flanking the QTL and away from the QTL were analysed by only SSR markers to know whether the recurrent parent is recovered or not (back ground selection) in the non-target region. Based on these SSRs and RFLP genotyping data, the individuals having 'H' or 'A' allele on the LG7 and 'B' allele on all other linkage groups, LG1 toLG6 were selected. And the selected progenies will be advanced by further selfing and/or backcrossing to next generation.

The target of this study is to provide early access to large straw samples of near-isogenic lines (NIL) and their hybrids for *in vivo* trials to permit assessment of the utility of this putative QTL in terms of livestock performance like milk production and live weight gains. Marker assisted backcross introgression of this putative stover quality QTL will soon provide near-isogenic lines for such *in vivo* and *in vitro* assessments. Hybrids of such lines will not only have high grain and stover yield potential and good resistance, but also increased stover quality in terms of its nutritive value for ruminant livestock without sacrificing grain yield.

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The pattern of "literature cited" is in accordance with the "guidelines" for thesis presentation for Acharya N. G. Ranga Agricultural University, Hyderabad.

# APPENDIX

## (a) SSR MARKERS AND THEIR CHARACTERISTICS

| Marker | LG | Primer pair (F/R) | PCR product | Repeat type and length | PIC value |
|---|---|---|---|---|---|
| 2273psmp | 1 | 5' AAC CCC ACC AGT AAG TTG TGC TGC 3'<br>5' GAT GAC GAC AAG ACC TTC TCT CC 3' | 169 | (GA)12 | 0.75 |
| 2201psmp | 2 | 5' CCC GAC GTT ATG CGT TAA GTT 3'<br>5' TCC ATC CAT CCA TTA ATC CAC A 3' | 364 | (GT)6 | 0.69 |
| 2225psmp | 2 | 5' CCG TAC TGA TGA TAC TGA TGG TT 3'<br>5' TGG GAG GTA AGC TCA GTA GTG T 3' | 250 | (GT)12 | - |
| 2231psmp | 2 | 5' TTG CCT GAA GAC GTG CAA TCG TCC 3'<br>5' CTT AAT GCG TCT AGA GAG TTA AGT TG 3' | 300 | (TG)12GG(TA)4 | 0.65 |
| 2059psmp | 2 | 5' GGG GAG ATG AGA AAA CAC AAT CAC 3'<br>5' TCG AGA GAG GAA CCT GAT CCT AA 3' | 119 | (AC)11 | 0.59 |
| 2267psmp | 3 | 5' GGA AGG CGT AGG GAT CAA TCT CAC 3'<br>5' ATC CAC CCG ACG AAG GAA ACG A 3' | 241 | (GA)16 | 0.79 |
| 2076psmp | 4 | 5' GGA ATA GTA TAT TGG CAA AAT GTG 3'<br>5' ATA CTA CAC CTG TAA GCA TTG TC 3' | 161 | (AC)15 | 0.59 |
| 2084psmp | 4 | 5' AAT CTA GTG ATC TAG TGT GCT TCC 3'<br>5' GGT TAG TTT GTT TGA GGC AAA TGC 3' | 245 | (AC)42 | 0.80 |
| 2202psmp | 5 | 5' CTG CCT GTT GAG AAT AAA TGA G 3'<br>5' GTT CCG AAT ATA GAG CCC AAG 3' | 161 | (GT)8 | 0.42 |
| 2270psmp | 6 | 5' AAC CAG AGA AGT ACA TGG CCC G 3'<br>5' CGA CGA ACA AAT TAA GGC TCT C 3' | 153 | (GA)26 | 0.80 |
| 2213psmp | 6 | 5' CCC AAA AGA ACC ACA CCC AC 3'<br>5' GTT GAT GCT ACT GCT CGT TTG 3' | 197 | (GT)10 | 0.10 |
| 2013psmp | 7 | 5' GTA ACC CAC TAA CCC TTA CC 3'<br>5' GTA ACC CAC TAA CCC TTA CC 3' | 153 | (CT)19(GT)16 | 0.88 |
| 2027psmp | 7 | 5' AGC AAT CCG ATA ACA AGG AC 3'<br>5 AGC TTT GGA AAA GGT GAT CC 3' | 273 | (GT)31 | 0.86 |

| Marker | LG | Primer pair (F/R) | PCR product | Repeat type and length | PIC value |
|---|---|---|---|---|---|
| 2043psmp | 7 | 5' TCA TAT TCT CCT GTC TAA AAC GTC 3'<br>5' ACA AAT CGT ACA AGT TCC ACT C 3' | 192 | (CA)13(GA)6 | 0.62 |
| 2019psmp | 7 | 5' TGT GCC ACA GCT TGT TCC TC 3'<br>5' CAA GCA GCC AGT TCC TCA TC 3' | 248 | (CA)38 | 0.85 |
| 2040psmp | 7 | 5' CAT TAC ACG TTT CTT CAA ACG C 3'<br>5' TCT TCG GCC TAA TAG CTC TAA C 3' | 163 | (CA)nd | 0.67 |

**(b) DESCRIPTION OF NUMERICALS IN THE TABLE (S)**

**Family P (17):**

There are a total of 17 BC5F1 families and each family has about 20-25 plants. The leaves of all plants in each family are collected and pooled (represented as 'P') for small scale DNA extraction. So each family DNA sample is named as 1 to 17 and SSRs analysis is done.

**Family 328(11):**

This is a BC4F2 family in which 11 plants are selected and small scale DNA extraction of all these 11 plants is done separately *i.e* not pooled for SSRs analysis.

**Family 330(20):**

This is a BC4F2 family in which 20 plants were selected for DNA extraction and SSRs analysis.

**Family 332(32):**

This family belongs to BC4F2 generation which has 32 plants. DNA is extracted from all the 32 individuals separately for SSRs analysis.

**Family 338(34):**

The family 338 belongs to BC4F2 generation in which 34 plants are present. Small scale DNA extraction (HTP method) SSRs analysis is done individually.

**Family 340(18):**

This is a BC4F2 family in which 18 plants are present and DNA is extracted for SSRs analysis.

**Family 342(33):**

The family 342 belongs to BC4F2 population/generation in which 33 plants are selected for SSRs analysis.