GENETIC DIVERSITY ANALYSIS IN RICE AND PEARL MILLET USING DNA MARKERS

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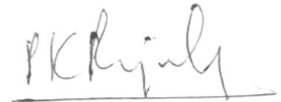## DOCTOR OF PHILOSOPHY

IN CHEMISTRY (BIOCHEMISTRY)

BY

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DECLARATION

Certified that the work incorporated in the thesis entitled "**GENETIC DIVERSITY ANALYSIS IN RICE AND PEARL MILLET USING DNA MARKERS**" submitted by Mr. **Kodavali Venkata Chowdari** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

(P.K. Ranjekar)

Research Guide

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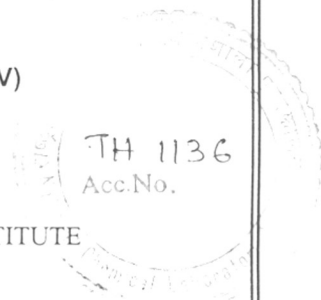PUNE, (M.S.) INDIA

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LIST OF ABBREVIATIONS

AFLP	:	Amplified Fragment Length Polymorphism
Bp	:	Base pairs
BSA	:	Bulk Segregant Analysis
cM	:	centi Morgan
CMS	:	Cytoplasmic Male Sterility
dATP	:	Deoxyadenosine 5' - triphosphate
dCTP	:	Deoxycytidine 5' - triphosphate
dGTP	:	Deoxyguanosine 5' - triphosphate
dTTP	:	Deoxythymidine 5' - triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
GD	:	Genetic Distance
GS	:	Genetic Similarity
IBPGR	:	International Board for Plant Genetic Resources
ICRISAT	:	International Crop Research Institute for Semi Arid Tropics
IRA	:	Inter Repeat Amplification
IRRI	:	International Rice Research Institute
Kbp	:	Kilobase pairs
MAS	:	Marker Assisted Selection
NIL	:	Near-Isogenic Lines
PCR	:	Polymerase Chain Reaction
QTL	:	Quantitative Trait Loci
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
SCAR	:	Sequence Characterised Amplified Region
SDS	:	Sodium Dodecyl Sulphate
SPAR	:	Single Primer Amplification Reaction
SSLP	:	Simple Sequence Length Polymorphism
SSR	:	Simple Sequence Repeats
STMS	:	Sequence Tagged Microsatellite Site
STR	:	Short Tandem Repeats
TAE	:	Tris-Acetate-EDTA buffer
Tm	:	Melting temperature
TPE	:	Tris-Phosphate-EDTA buffer
Tris	:	Tris-hydroxymethyl amino methane
VNTR	:	Variable Number of Tandem Repeat

CHAPTER I

APPLICATION OF DNA MARKERS IN GENETIC DIVERSITY ANALYSIS AND PREDICTION OF HYBRID PERFORMANCE

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APPLICATION OF DNA MARKERS IN GENETIC DIVERSITY ANALYSIS AND PREDICTION OF HYBRID PERFORMANCE

1.1 INTRODUCTION

One of the major objectives of the crop improvement program is to develop new genotypes with better traits such as high yield, disease resistance, good quality etc. For such development or improvement of plant varieties, conventional plant breeding involves several methods such as crossing, backcrossing, selfing and testing wherein, two parents having desirable characteristics are crossed, in order to either transfer a particular character from one parent to another, following which, the gene introgression is studied by backcrossing and selfing or to select progeny having desirable characteristics of both the parents which are stabilised by further selfing. A number of cultivars have been developed from wild species, and a number of superior genes have been transferred to the cultivated crops over the past years and the process continues to date. Methods that would assist the efforts of the breeders, so as to accelerate the breeding programs have always been desirable and a lot of effort is being made to this effect over the past decade.

Primarily the success of crop improvement program depends upon the genetic diversity present in the genotypes under consideration. Established methods for the characterization and measurement of genetic diversity have mostly relied on morphological, physiological and cytological markers as well as biometrical analysis of quantitative variation. The analysis of genetic diversity between or within different species, population and individuals has played a key role in plant breeding programs. A number of complications are faced when classical strategies are used to characterize genetic diversity. Hence during the past decade, the classical strategies are being complimented by molecular markers involving macromolecules like proteins and DNA. These markers, which are based on the polymorphism found in the proteins and DNA, have revolutionised the genetic analysis methods in crop plants.

Isozymes have been exploited as molecular markers for variability analysis and construction of framework genome maps. However, there have

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been limitations in their use as markers for classification of breeding materials, because of the small number in which they are available.

DNA markers obviate these limitations and are extensively used for various applications in plant breeding procedures. The DNA markers tightly linked to various qualitative and quantitative traits can be used in Marker Assisted Selection (MAS) in breeding program.

In the present chapter, various types of DNA markers, their utility in genetic diversity analysis and prediction of heterotic combinations in important hybrid crop breeding programs and finally the future directions of MAS breeding programs are discussed.

1.2   DNA MARKERS

Molecular markers are ubiquitous, innumerable, discrete, nondeleterious, inherited by Mendelian laws, unaffected by environment, and are free of epistatic interactions (Beckman and Soller 1986; Tanksley *et al* 1989). Today, various types of DNA markers and breeding strategies have been employed for the identification and utilization of agronomically important traits in many crop systems. The basic information and potential of various types of DNA markers such as Restriction Fragment Length Polymorphism (RFLP), microsatellites, minisatellites and PCR-based markers are described.

1.2.1 Restriction Fragment Length Polymorphisms (RFLPs)

RFLP is a result of differences in the nucleotide sequence in the DNA of two different genotypes. These differences are the result of mutations and rearrangements which occur during the evolution and are detected as variations (polymorphism) in the length of restriction fragments. RFLPs are co-dominant markers and are very reliable in linkage analysis and breeding. They can be used to study the nature of linked traits i.e. hetero or homozygous state, and this information is highly desirable, especially for recessive traits. Single copy genomic DNAs or cDNAs have been used as molecular genetic markers to construct RFLP maps of crop plants such as

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rice (Mc Couch *et al* 1988; Saito *et al* 1991), maize (Helentjaris 1987), lettuce (Landry *et al* 1987), soybean (Keim *et al* 1990), oil seed (Diers *et al* 1996), tomato (Young and Tanksley 1989), potato (Gebhardt *et al* 1989) and wheat (Chao *et al* 1989).The application of these maps and DNA probes in breeding research has been demonstrated for parental evaluation in F1 hybrid maize breeding (Walton and Helentjars, 1987), elucidation of QTLs in tomato (Paterson *et al* 1988) and tagging resistance genes [Sarfatti *et al* 1989, Klein-Lankhorst *et al* 1991, Barone *et al* 1990].

Genetic maps based on molecular markers have many further applications in plant breeding. New dense genetic maps developed by these markers have also found application in the resolution of some of the outstanding questions from classical genetic studies such as demonstrating intergenomic relationships between maize and sorghum (Hulbert *et al* 1990); and between tomato and potato (Bonierbale *et al* 1988, Gebhardt *et al* 1991). It is likely that there will be enough conservation of syntenic blocks so that genes located in one species will have the same flanking markers in another species. Comparative mapping of different crop plants (Bonierbale *et al* 1988, Tanksley *et al* 1988) will provide useful information about the location of important genes and will be a valuable tool for phylogenetic analysis, as well as in introgression studies.

Despite their usefulness, generation of RFLP markers and their application is time-consuming and expensive.

1.2.2 Microsatellites and minisatellites

Microsatellites are tandem repeats with a basic motif of less than 6 bp and are also called as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STR) (Jacob *et al* 1991). The repeat number of microsatellites has been demonstrated to be highly variable in animals and to be inherited in a codominant manner (Litt and Luty 1989; Johansson *et al* 1992). The presence of microsatellites has been documented in many plants such as Arabidopsis (Bell and Ecker 1994), barley (Saghai-Maroof *et al* 1994),

brassica (Lagercrantz *et al* 1993), maize (Condit and Hubbell 1991; Senior and Heun 1993), rice (Wu and Tanksley 1993), soybean (Akkaya *et al* 1992; Morgante and Olivieri 1993) and wheat (Roder *et al* 1995). Microsatellite molecular marker linkage maps developed in crops such as barley, wheat, rice,soybean and arabidopsis are gaining more importance in recent years. The physical distance of 1cM in plants ranges roughly from 150Kb (Arabidopsis), 300Kb (Rice), 500Kb (Tomato), 1000Kb (Potato) to 1500Kb (Maize) (Wang *et al* 1994a). Given the frequency of $(AT)_n$ every 62Kb, it would be expected to find, on an average, at least 2.5 (AT)n STRs (short tandem repeats) within 1cM for a simple genome like that of Arabidopsis (Wang *et al* 1994a). STRs of di-, tri- and tetranucleotide repeats should themselves be in sufficient numbers in order to be a reservoir of genetic markers in plants for linkage mapping and even for STS physical mapping. The apparent strand-slippage which occurs during PCR amplification of dinucleotide repeat STRs (Smeets *et al* 1989; Luty *et al* 1990) is significantly diminished with increasing repeat length (Economou *et al* 1990; Zuliani and Hobbs 1990).

Telomeric repeats, which are found at the ends of eukaryotic chromosomes, represent a rare example of simple sequences having clearly defined functions. They protect the chromosomal ends from degradation and fusion processes and compensate for DNA loss due to incomplete replication of chromosomal ends. A telomeric repeat which is conserved in a variety of eukaryotes has been shown to detect polymorphism in closely related cultivars of tomato and maize (Broun *et al* 1992).

Minisatellites comprise a class of variable number of tandem repeat (VNTR) loci in which the repeated sequences are short (<65bp) and frequently GC rich (Jeffreys *et al* 1985a; 1985b; Nakamura *et al* 1987). The presence of numerous, dispersed, highly polymorphic minisatellites appears to be an ubiquitous feature of eukaryotic genomes (Ryskov *et al* 1988). Minisatellites have been widely used in humans and various animal species to yield DNA 'fingerprints' which are unique to each individual. A 15 bp repeat motif in the protein III gene of bacteriophage M13 appears to be extremely

useful in detecting polymorphism (Vassart *et al* 1987) and serves as an universal marker for DNA fingerprinting (Ryskov *et al* 1988). It has been used for the first time in plants by Ryskov *et al* (1988) to identify polymorphic regions in barley, to detect minisatellite-like sequences in a few gymnosperms and angiosperms (Rogstad *et al* 1988; Zimmerman *et al* 1989) and to assess genetic variability in Rosaceae (Nybom *et al* 1990) and Ornamental plants (Tzuri *et al* 1991). Jeffrey's 33.6 and 33.5 minisatellite probes have been used for fingerprinting cultivars of Asian and African rice (Dallas 1988) while a human minisatellite probe, pV47 has been shown to be polymorphic in indica, japonica and wild rice (Ramakrishna *et al* 1995).

1.2.3 PCR-based markers

PCR is a versatile technique and has revolutionized research in many areas of molecular biology. PCR with arbitrary primers and tandem repeat primers is shown to be useful in DNA fingerprinting of various crop plants. I would like to summarize the various PCR derived methods in this section of DNA markers.

RAPDs (Random Amplified Polymorphic DNA)

RAPD markers, obtained by using single primer of arbitrary nucleotide sequence in Polymerase chain reaction (PCR) amplification, have been developed by Williams *et al.*(1990) and Welsh and McClelland (1990) as molecular markers for use in genetic analysis. Since RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region (Williams *et al* 1990), these polymorphisms are usually noted as presence or absence of an amplification product from a single locus. This also means that the RAPD technique tends to provide only dominant markers.

RAPDs have been shown to be useful in genetic mapping (Martin *et al* 1991; Reiter *et al* 1992; Barua *et al* 1993; Paran and Michelmore 1993; Williams *et al* 1993), genetic fingerprinting (Quiros *et al* 1991; Wilde *et al*

1992; Collins and Symons 1993; Graham *et al* 1994), phylogenetic analysis (Demeke *et al* 1992; Halward *et al* 1992; Kresovich *et al* 1992; Kazan *et al* 1993; Yu and Pauls 1993) and parentage analysis (Welsh *et al* 1991a; Roy *et al* 1992; Scott *et al* 1992). One of the first practical uses of RAPD markers is in the creation of high-density genetic maps. For example, Reiter *et al* (1992) have been able to place over 250 new genetic markers on a recombinant inbred population of *A. thaliana* in a short span of time, clearly demonstrating the utility of RAPD markers for quickly saturating both global and local genetic maps.

Several groups have used the RAPD-assay as an efficient tool to identify molecular markers that lie within the region of a genome introgressed during the development of near isogenic lines (Klein-Lankhorst *et al* 1991; Martin *et al* 1991; Paran *et al* 1991). Michelmore *et al* (1991) have described the use of RAPD technique for efficient screening of markers linked to specific regions of the genome by Bulk Segregant Analysis (BSA).

In spite of numerous advantages, RAPDs have an inherent disadvantage of reproducibility amongst laboratories because of the reaction conditions i.e. low annealing temperature, ionic conc. and template quality.

DAF (DNA Amplification Fingerprinting)

DAF involves enzymatic amplification of DNA directed by a single arbitrary oligonucleotide primer (Caetano - Anolles *et al* 1991). DAF can be used to generate complex and characteristic fingerprints. Each amplification product reflect single base pair changes in a particular primer-target site making DAF a powerful fingerprinting strategy, especially for organisms that are closely related.

SCAR (Sequence Characterized Amplified Region)

This technique, developed by Paran and Michelmore (1993), is derived from RAPD and circumvents the drawbacks inherent to RAPDs. The markers are generated by cloning and sequencing RAPD fragments of interest and

designing 24-mer oligonucleotide primers that are complementary to the ends of the original RAPD fragment. Using these primers, single locus called SCAR can be specifically amplified. These SCARs have more advantages compared to RAPDs because of the reproducibility and co-dominant nature and can be used in map-based cloning, physical mapping and comparative mapping.

STMS (Sequence Tagged Microsatellite Site)

Microsatellite DNA sequences are an excellent source of polymorphisms in eukaryotic genomes and are well suited for genotyping and map construction. Polymorphisms appear because of variation in the number of tandem repeats in a given repeat motif at a particular locus. Most SSRs are dinucleotide repeat-based [$(AT)_n$, $(AG)_n$ and $(AT)_n$] microsatellite markers (Rafalski and Tingey 1993). Such polymorphisms are amplified by designing primers from the sequenced regions flanking the repeat motifs. Similar to $(CA)_n$ repeats in humans, $(AT)_n$ dinucleotide microsatellite repeats are relatively abundant and highly polymorphic in plants (Akkaya et al 1992; Morgante et al 1993). These markers are inherited in a mendelian fashion and can be used for segregation and linkage analyses. In recent years, PCR amplification of microsatellites has been successfully applied to the analysis of plant genomes (Morgante and Olivieri 1993; Wu and Tanksley 1993; Panaud et al 1996, Chen et al 1997). The usefulness of microsatellite markers in crop improvement can be expected to increase dramatically as saturated genetic Simple Sequence Length Polymorphic (SSLP) maps become available.

IRA (Inter Repeat Amplification)

IRA is performed using a single, long PCR primer at high stringency which improve reproducibility compared to RAPDs. IRA bands appear to be dominant like RAPDs (Zietkiewicz et al 1994). A disadvantage compared to microsatellites is that genetic interpretation of the polymorphisms may be more complex than for single locus STS markers.

SPAR (Single Primer Amplification Reaction)

SPAR is a DNA marker system that can provide multiple markers per assay. The system uses primers based on microsatellites or simple sequence repeats (SSRs) and amplifies inter-SSR DNA sequences (Gupta *et al* 1994). Of di-, tri-, tetra-, and pentanucleotide SSRs, tetranucleotide repeats are most effective in producing polymorphic multiple banding patterns.

AFLPs (Amplified Fragment Length Polymorphisms)

AFLPs are based on selective amplification of restriction enzyme digested DNA fragments with specific primers (Vos *et al* 1995). Multiple bands are generated in each amplification reaction that contains DNA markers of random origin. Analysis of DNA on denaturing polyacrylamide gels typically results in the production of 50 to 100 bands per individual sample. Similar to RAPD analysis, AFLP assay requires no prior sequence knowledge, but detects a 10-fold greater number of loci than those detected by RAPD analysis. Thus, the AFLPs have the capacity to rapidly screen thousands of independent genetic loci. Because of this nature, it will have added advantage when combined with Near-Isogenic Lines (NILs) or Bulk Segregant Analysis (BSA). These markers are gaining more importance in developing saturated genetic linkage maps in various crop plants.

Eventhough different DNA markers have a good potential in plant breeding, the final choice of markers depends on requirements/purpose for which these are to be used in addition to features such as reproducibility, information content, developing cost, automation and proprietary rights status. Table 1 (Staub *et al* 1996) gives comparisons among several molecular marker systems for various technical attributes.

Table 1 Comparisons among several molecular marker systems for various technical attributes (Staub et al 1996).

Critical variables	Molecular marker systems[1]							
	RFLP	AFLP	RAPD	SCAR	DAF	SSR	IRA	SPAR
Tissue sampling (weeks)[2]	4	2	2	2	2	2	2	2
DNA needed/100 markers (mg)	100	0.5	2.5	2.5	2.5	10	2.5	2.5
Cloning and sequencing	yes	No	No	Yes	No	yes	No	No
Information content per run[3]	0-3	0-30	0-4	0-2	0-10	0-2	0-10	0-20
Marker type[4]	C	D	D	C	D	C	C	D
Zygosity detection[5]	yes	No	No	yes	No	yes	yes	No
Automation[6]	+	+++	++	++	++	+++	++	++
Utility of genetic maps[7]	SS	CS	CS	CS	CS	SS	CS	CS

[1]RFLP = restriction fragment length polymorphism, AFLP = amplified fragment length polymorphism, RAPD = random amplified polymorphic DNA, SCAR = sequence characterized amplified region, DAF = DNA amplification fingerprinting, SSR = simple sequece repeats, IRA = inter repeat amplification and SPAR = single primer amplification reaction.
[2]The sampling time after sowing is shown for corn (Zea mays) (relative time applies to all crop species).
[3]Markers obtained per hybridization or PCR reaction. For SSR markers per run does not reflect multiplexing.
[4]D and C equal dominant and codominant markers, respectively.
[5]Heterozygous alleles can be distinguished from the homozygous alleles.
[6]On the scale of 1 to 3, + = the least and +++ shows the most potential for automation.
Automation refers to mechanizing steps involving processing of DNA and detection, identification and scoring of markers.
[7]Refers to the relative utility of maps constructed with a given marker system either within that species (SS=species specific) or to a specific cross or population (CS=cross specific).

1.3 GENETIC DIVERSITY ANALYSIS

So far I have discussed various DNA markers and their potential applications in crop breeding programs. Now, I would like to emphasize the importance of genetic diversity studies in developing varieties and preserving the genetic resources. The modern agricultural practices to develop varieties are so far successful, but there is a reduction in the genetic diversity of the primary gene pool under cultivation. This will lead to sporadic breakdown of the crop systems due to their increasing susceptibility to pathogens. Hence awareness of genetic diversity and management of crop genetic resources are both important components in plant improvement programs.

1.3.1 Germplasm collection

The foundation of crop-based agriculture largely rests on the availability and knowledge of exotic plant genetic resources in germplasm collections. Genetic conservation is being carried out at international, national and institutional levels. The International Board for Plant Genetic Resources (IBPGR) was established in Rome in 1973 for the purpose of having liaison between these conservation programs and to support their activities. Later on, many international centers were established to maintain germplasm of various crops. The International Rice Research Institute (IRRI) maintains the international gene bank for rice in its Rice Germplasm Center, which has about 83,000 accessions. Large collections of rice germplasm are also conserved in national gene banks in India, Thailand, China, Indonesia, Japan and several other countries. Collections of wild rice germplasm are being maintained at IRRI and at the National Institute of Genetics, Mishima, Japan (Khush *et al* 1991).

Systematic collection of pearl millet germplasm in India was undertaken by the Rockefeller Foundation in 1959 in collaboration with the Indian Agricultural Research Institute, New Delhi. A total of 22,056 pearl millet accessions were assembled at ICRISAT from 27 different countries

(Mengesha and Appa Rao, 1982). The IBPGR has summarized information on the world collections of pennisetum (Archeampong *et al* 1984).

Establishment of a core collection based on genetic variation shown by accessions will be advantageous, but clear and detailed assessments of diversity within germplasm collections are not usually available. This information can be obtained using molecular markers. These markers have the advantage of sampling at the genome level directly. To assess variation many reports are available in plants using various markers such as RFLPs (Helentjaris *et al* 1985; Tanksley *et al* 1989), RAPDs (Fukuoka *et al* 1992; Yu and Nguyen 1994), microsatellites (Akkaya *et al* 1992), DAF (Caetano - Anolles *et al* 1991), SPARs (Gupta *et al* 1994) etc. Information on the extent and distribution of diversity will assist in the development of efficient collection and sampling strategies and in the identification of locations as centers of diversity.

1.3.2 Elite / landraces / wild germplasm

The recent modernization of agriculture and the establishment of a monoculture of a few profitable varieties has resulted in genetic erosion and increased risk of outbreak of diseases due to bacteria, fungi and insects. A large number of important alleles were lost during development of elite varieties. As a result many improved varieties are interrelated genetically and often carry the same genes for disease and insect resistance.

The existence of genetic diversity enables crop plants to evolve and cope with environmental changes. Landraces generally have low yield capacity but yield levels are highly stable. They may be regarded as natural composite varieties that have an array of resistance genes, and are well buffered so that no single race or biotype of plant pests can attack at the time of epidemic. In rice, of the large collection kept at IRRI, some 9,700 or about 12% of the total species/genotypes, have useful genes for tolerance to drought, cold, deepwater, adverse soils, disease resistance , insects and nematode resistance. A gene for resistance to grassy stunt virus of rice was

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found in *O. nivara* (= *O.rufipogan*) and transferred to *O.sativa* by back crossing (Khush *et al* 1977). Similarly, a gene for resistance to many races of bacterial blight of rice was found in *O.longistaminata* and transferred to *O.sativa* by back crossing (Khush *et al* 1990). In case of resistance, the probability of finding an useful gene is higher in wild germplasm than among ordinary cultivars (Table 2). This indicates the importance of wild species, particularly the wild relatives of cultivars, in conservation programs.

The DNA markers make it possible to understand the processes and dynamics of biodiversity, its evolution and natural preservation in cultivars, landraces and wild species (Helentjaris *et al* 1985; Milligan *et al* 1994; Lande 1988). Marker-based selection is helpful in attempts to transfer genes from exotic germplasm into cultivated lines. Molecular-genetic maps can be used to exploit the genetic potential of wild species for the improvement of yield and quality in modern plant cultivars (Tanksley *et al* 1996; Xiao *et al* 1996a).

Table 2 Proportion of accessions resistant to insects in *O. sativa* cultivars and wild species of *Oryza* (Heinrich *et al* 1985)

Insect	Cultivars		Wild species	
	Tested (no.)	Resistant (%)	Tested (no.)	Resistant (%)
Brown planthopper (*Nilaparvata lugens*)				
Biotype 1	30,790	0.91	248	48.0
Biotype 2	8,922	1.41	248	39.1
Biotype 3	10,711	1.38	248	39.5
Whitebacked planthopper (*Sogatella furcifera*)	46,488	0.84	228	37.7
Green leafhopper (*Nephotettix virescens*)	47,944	2.50	288	49.1
Zigzag leafhopper (*Recilia dorsalis*)	2,370	1.52	208	55.2
Striped stem borer (*Chilo suppressalis*)	15,000	0.15	167	4.2
Yellow stem borer(*Scripophaga incertulas*)	19,961	0.13	181	13.3
Leaffolder (*Cnaphalocrocis medinalis*)	17,914	0.20	264	3.03
Whorl maggot (*Hydrellia philippina*)	15,787	0.01	328	2.13

1.3.3 Identification / classification / management.

The system of management of core collections is more important than the total number of accessions kept in a gene bank. Germplasm management is a multifaceted endeavor involving acquisition, maintenance, and characterization such that the plant genetic resources are conserved and utilized for crop improvement. When the size of collection increases, it becomes more difficult to avoid the inclusion of duplicate or at least very similar accessions. Evaluation of numerous, highly similar accessions not only wastes plant breeding resources but also reduces the chance of identifying the truly unique and valuable accessions. The best sampling strategies might increase the efficiency and effectiveness of identifying and utilizing germplasm collections in plant breeding programs.

The DNA markers can be effectively used in identification, classification and managing the germplasm collections (Virk *et al* 1995; Howell *et al* 1994; Kazan *et al* 1993; Fukuoka *et al* 1992). The prospect of utilizing DNA marker technology for managing germplasm collections is reviewed by Kresovich and McFerson, 1992 and Bretting and Widrlechner,1995. However, this technology has not yet made a significant impact on the management and utilization of germplasm collections.

1.4   HYBRID PERFORMANCE (HETEROSIS)

Heterosis (Hybrid vigor) term was coined by G.H.Shull (1914), describing the superiority of F1 performance over some measure of the performance of its parents. Several investigations have been carried out but the genetical, physiological and biochemical bases of this phenomenon still remain largely unexplained. As Frankel (1983) stated **"the causal factors for heterosis at the physiological and biochemical level are today almost as obscure as they were 30 years ago".**

1.4.1 Heterosis phenomenon

The heterosis phenomenon has been exploited by breeders to enhance the productivity of numerous crops and horticultural plants, and the effects of the phenomenon have been quantified in a wide variety of plant studies. Inspite of the many attempts that have been made so far, there has not been a satisfactory explanation for the genetic cause of heterosis. Two principal hypotheses have been put forward initially and though each has its own drawbacks, they hold good until now. Several modifications, of course, have been suggested from time to time. The two hypotheses are (i) the dominance hypothesis and (ii) the over-dominance hypothesis.

(i) Dominance hypothesis: The assumptions in this hypothesis are :

a) Genes governing vigour are beneficial and dominant in nature whereas recessives are deleterious and have detrimental effect on heterosis.

b) There is no non-allelic interaction of alleles and there is complete additivity of dominant gene effects.

(ii) Over-dominance hypothesis : This was proposed independently by Shull (1908) and East (1908) and later advocated by Stadler (1939), Gustafson (1938) and Hull (1945). The evidence for true overdominance (i.e. Single locus at which two alleles have the property that the heterozygote is truly superior to either homozygote) is very limited, particularly in plants, and usually cannot be distinguished from psudo-overdominance. Pseudo-overdominance (i.e. nearby loci at which alleles having dominant or partially dominant advantageous effects are in repulsion phase linkage) is an extension of the dominance of linked factors hypothesis put forth by Jones (1917). In this case, the linked block of two or more loci tends to respond as a single locus and the effect is nearly impossible to distinguish from true overdominance.

There is some evidence that interaction of non alleles (epistasis) may be an important factor in the type of heterosis found in many maize single crosses (Otsuka *et al* 1972; Stuber *et al* 1973). Schnell and Cockerham (1992) have reviewed the influences exerted on heterosis by multiplicative effects between genes (a specific type of epistasis). Heterosis in quantitative traits may arise from action and interaction of polygenes and such interactions can be inter-allelic, and non-allelic. Available biometrical techniques have failed to precisely differentiate among such interactions. Hence, no single genetic theory can explain the entire expression of heterosis. For complex traits, heterosis must be conditioned by numerous genetic factors functioning and responding to a wide variety of interactive situations. With the use of modern molecular tools, there is now some hope that the dissection of this phenomenon into the individual components may be possible.

1.4.2 Measures of genetic similarity based on pedigree

Malecot (1948) has defined the coancestry coefficient 'f' as the probability that two homologous genes drawn at random, one from each of the two individuals, are identical by descent for the quantification of genetic similarity between two related individuals. The 'f' values have been used in some autogamous crops such as barley, oats, wheat and soybean to (i)determine the genetic relationships between cultivars of a gene pool adapted to a certain region (ii)compute the relative genetic contributions of ancestral genotypes to released cultivars and (iii) examine the level of genetic diversity of a given gene pool and monitor its changes over a time.

The coancestry coefficient is an indirect measure of genetic similarity and its application is questionable due to unrealistic assumptions (absence of selection and genetic drift) underlying the calculation of 'f' values (Cox and Murphy 1990; Smith *et al* 1990).

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1.4.3 Measures of genetic distance based on molecular markers

Molecular markers have been advocated as a means to assess precisely genomic diversity. RFLPs and microsatellites have been utilized as a measure of genetic diversity in studies targeting the relationship between genetic diversity and heterosis (Lee et al 1989; Melchinger et al 1990b; Smith et al 1990; Zhang et al 1994 to 1997). RAPDs have shown some promise as molecular markers in genetic diversity studies (D'Ovidio et al 1990; Devose and Gale 1992; Xiao et al 1996b).

All assays of molecular markers currently used (isozymes, RFLPs, microsatellites and minisatellites and PCR-based markers) generate a characteristic banding pattern for each individual. Each banding pattern can be assigned a marker genotype and estimation of genetic distances (GD) can be calculated from the differences in allele frequencies at the marker loci. When the banding pattern is complex and the genotype cannot be determined directly, it is a common practice to calculate a GD estimate by determining the fraction of bands that are not common between the two individuals considered (Nei and Li, 1979). Phylogenetic trees derived from RFLP data have agreed well with previous classifications based on biosystematic studies and / or chemotaxonomy (Miller and Tanksley, 1990; Debner et al, 1990). Crucial considerations for the application of molecular markers for grouping of genotypes are :

i) The mean genetic similarity among genotypes within germplasm groups and between germplasm groups.

ii) The variation in genetic similarity of genotypes from the same as compared to different germplasm groups.

iii) The establishment of simple and robust criteria that allow unambiguous assignment of genotypes (with unknown genetic background) to the respective germplasm group.

1.4.4 Relationship of genetic distance with hybrid performance

Experimental studies (Moll *et al* 1965) illustrated that midparent heterosis could be related to genetic divergence, a relationship that was supported by quantitative genetics theory (Falconer 1981). Distances computed from isozyme loci data were in some cases significantly correlated to heterosis, but the correlations were generally too low for the distances to be of practical predictive value (Peng *et al* 1988). Insufficient genome coverage, due to the low number of marker loci, was a possible explanation for these results. Later on, RFLPs and RAPDs were used in several crops and prediction of hybrid performance was shown to be highly dependent on the germplasm involved in the study [maize (Lee *et al* 1989; Godshalk *et al* 1990; Melchinger *et al* 1990a, b; Smith *et al* 1990; Dudley *et al* 1991; Melchinger *et al* 1992; Boppenmeirer *et al* 1992); Brassica (Diers *et al* 1996); rice (Zhang *et al* 1992, 1994, 1995, 1996) and wheat (Martin *et al* 1995)]. From these results, it is indicated that the correlation between genetic distance and hybrid value increases with increasing relatedness in the germplasm under study. Melchinger *et al* 1992 have concluded that RFLP distances would not be useful for predicting the value of crosses between lines that belong to different heterotic groups in maize inbreds and this conclusion was supported by Boppenmeier *et al* (1992).

In conclusion, it appears that GS estimates based on either RFLPs or f (coancestry coefficient) values have only limited potential for correlation with hybrid performance.

1.4.5 QTLs associated with yield components

The advent of molecular marker technology has led to the development of genetic maps that make it possible to identify and locate genes involved in quantitative trait loci (QTLs) controlling quantitative characters. Yield component, is a typical example for a quantitative trait. Determination of the number, location and magnitude of effects through RFLP linkages of QTLs underlying yield component could elucidate the genetic

basis of the trait leading to improved breeding and selection efficiency (Stuber et al 1992; Paterson 1995a).

The heterosis phenomenon can be studied in detail with the advent of recent molecular linkage maps which can detect and individually analyze the loci which are controlling yield. Using molecular markers, Stuber et al (1992) are able to detect quantitative trait loci (QTLs) contributing to hybrid vigor in maize. In this study, the heterozygotes of most QTLs detected for grain yield have higher phenotypic values than those of either respective homozygotes. This indicates that overdominance is the principal factor controlling heterosis in open-pollinated crop species.

Xiao et al (1995) have shown that dominance is the major genetic basis of heterosis in rice by analyzing the QTLs using molecular markers. Recently, Yu et al (1997) have shown that epistasis is the genetic basis of heterosis in an elite rice hybrid. In their study, correlations between marker heterozygosity and trait expression are low, indicating that the overall heterozygosity has made little contribution to heterosis. Thus the heterosis phenomenon is very complex than the expectations based on dominance and overdominance hypotheses.

Comparative mapping is also useful in molecular dissection of quantitative traits. A close correspondence among QTLs affecting complex traits such as seed size, as well as traits of varying complexity in different taxa such as shattering of the inflorescence and day neutral flowering, has been shown for sorghum, sugarcane, maize, wheat, barley and rice (Paterson et al 1995b). Correspondence among an unexpectedly high number of genes affecting height and flowering of maize, sorghum and other grasses has also been reported (Lin et al 1995).

The identification of multiple independent mutant alleles at QTLs would permit testing of correlation between phenotype and mutation in a particular transcript, as has been used in map based cloning of genes in humans and other organisms (Robertson 1985; Paterson 1995a). Identification of discrete

mutations corresponding to QTLs offers a more rapid and efficient means to dissect quantitative traits (Paterson *et al* 1990; Dorweiler *et al* 1993).

1.5 FUTURE PROSPECTS OF MARKER ASSISTED SELECTION (MAS) IN CROP BREEDING PROGRAMS

Gene introgression requires identification of the desired genotypes in each generation. Frequently, this identification is not simple due to either genetic reasons (recessiveness of the desired alleles) or non genetic factors such as low heritability of quantitative traits. The use of genetic markers in marker assisted selection (MAS) (Tanksley and Rick 1980; Soller and Beckmann 1986) has been examined and analyzed for such purpose. Marker-assisted selection (MAS) provides a potential for increasing selection efficiency by allowing for earlier selection and reducing plant population size used during selection. The prediction value of genetic markers used in MAS depends on their inherent repeatability (Weeden *et al*, 1992), map position and linkage with economically important traits (quantitative or qualitative). The presence of a tight linkage (<10cM) between quantitative trait(s) and genetic marker(s) may be useful in MAS to increase gain from selection (Kennard *et al*, 1994, Paran *et al*, 1991, Timmerman *et al*, 1994). Selection for multiple loci or Quantitative Trait Loci (QTL) using genetic markers can be effective if a significant association is found between a quantitative trait and markers (Edwards and Page 1994; Edwards *et al*, 1987; Lande and Thompson, 1990).

It is evident that the development of DNA markers has revolutionized the construction of genetic maps in plants and the utilization of genetic maps in studies of plant evolution, systematics, and practical applications such as plant breeding. The utility of molecular marker maps will continue to increase. The ability to construct genetic maps rapidly has made possible applications that were unthinkable using conventional mapping techniques. Technology for the utilization of DNA markers is evolving rapidly at the present time and further advances are sure to occur soon. Some of these will involve making

the process of developing and utilizing DNA markers technically simpler, less expensive and more capable of automation.

1.6 GENESIS OF THESIS

In the present thesis, I have carried out work on rice and pearl millet with special emphasis to two aspects, namely

(I) Assessment of genetic variability and DNA fingerprinting of Indian elite rice cultivars, pearl millet cultivars and pearl millet landraces.

(Ii) Comparison of molecular divergence with field performance of CMS and restorer lines of rice and pearl millet to predict heterotic combinations.

The genesis of this work is as follows :

The Rockefeller Foundation which supports Rice Biotechnology research, granted program to NCL on 'DNA markers in Hybrid rice program' in 1994. The main emphasis of the project is on the characterization of Indian elite rice cultivars and use of molecular markers in hybrid rice program in collaboration with Directorate of Rice Research, Hyderabad. Indian elite rice cultivars developed since 1960's relied mostly on a few parents. There was no systematic study at the genetic level which prompted me to take this work to study molecular divergence with DNA markers.

Dr. O.P. Govila, head of the All India Pearl Millet Improvement Program, visited NCL in 1994 and gave information regarding the valuable material available at IARI and with his interest in pearl millet molecular work, the collaborative research program was initiated. The pearl millet cultivars developed by breeders are from a narrow genetic base yet showed very distinct characteristics. Being a cross-pollinated crop this is possible and precise estimation of genetic variation in these cultivars will be useful to develop further varieties. Dr. K.E. Prasad Rao, Genetics Resources Division, ICRISAT, Hyderabad has kindly provided the landraces seed material and encouraged me to carry out the genetic diversity studies.

Heterosis is a complex phenomenon and use of molecular markers for this aspect will be challenging. The research work carried out by Prof. Qifa

Zhang, Prof. CW. Stuber, Dr. M. Lee and Dr. SD Tanksley in the area of heterosis made me curious to take up this work in our laboratory. The CMS and restoration system is very popular in India to develop hybrids and there were no reports on the usefulness of molecular markers in evaluating these lines in hybrid breeding program. For the application of DNA markers in prediction of heterotic combinations the two important crops in India, rice and pearl millet, which have two different pollination systems, self and cross, were selected.

CHAPTER II

RICE

In this section, emphasis is given to study the genetic architecture of Indian elite rice varieties using DNA markers. Based on these data the genetic relationship was also evaluated. Such information has relevance in the present status of rice improvement program. An attempt is made for prediction of heterotic combinations in hybrid rice breeding using DNA markers and discussions are made on the practical predictive value of this study.

CHAPTER II

SECTION - A

GENETIC DIVERSITY EVALUATION OF INDIAN

ELITE RICE VARIETIES USING RAPD MARKERS

The contents in this chapter have been communicated as a full paper to Plant Breeding.

INTRODUCTION

Rice, the most important crop, is grown all over India with a wide range of selected cultivars. Several rice varieties that have been developed by many rice research stations in India are mostly region specific. Self-sufficiency and reasonable stability in rice production in India are made possible by the development and widespread cultivation of plant-type based, high yielding varieties. Even then plateauing of yields in areas of assured irrigation during the last two and a half decades has been reported in India (Virmani 1994). This may be because of the narrow genetic base of the released rice varieties (Carmona 1990). Similar reports of the narrow genetic base of cultivated rice varieties are available from several other regions : Japan (Kaneda 1985), USA (Dilday 1990), Taiwan (Lin 1991) and Latin America (Cuevas-Perez et al 1992). Breeding strategies that involve broadening of the genetic base increase grain yield. Genetic diversity evaluation and identification of elite rice cultivars instead of local varieties which are low yielding will augment the production of rice in India. Maximizing yield in low productivity areas and exploring ways to raise the present potential yield areas are considered to be rational strategies for achieving the production goals.

New techniques based on DNA profiling offer advantages over traditional morphological comparisons. The development and application of randomly amplified polymorphic DNA (RAPD) markers generated by the Polymerase chain reaction (PCR) using arbitrary primers has led to alternative molecular markers for the detection of DNA polymorphisms (Williams et al 1990). These markers have been extensively used for cultivar identification (Hu and Quiros 1991), phylogenetic and pedigree studies (Kazan et al 1993; Scott et al 1992), and genetic mapping (Welsh et al 1991b). In rice, for example, RAPD markers have been used for genetic diversity evaluation in upland and lowland varieties (Yu et al 1994: Guimaraes et al 1996), identification and parentage determination (Wang et al 1994b; Fukuoka et al 1992), classification of Japonica cultivars

(Mackill 1995)and identification of duplicate accessions in the germplasm (Virk *et al* 1995). No such studies are, however, available on Indian rice genotypes except for our earlier reports where we have shown that micro- and minisatellite DNA sequences are very useful for DNA fingerprinting of cultivated and wild rice species (Ramakrishna *et al* 1994,1995).

The present study was undertaken to quantify the genetic diversity based on RAPD markers in a large sample of rice elite cultivars developed from 1968 to 1994 in India. Based on our results, we consider that RAPD markers will be very useful to identify and estimate relationships in cultivars that have been developed using a few parents repeatedly in the breeding program.

MATERIALS AND METHODS

Plant material : Rice cultivars used in this study (Table 1) were collected as seeds from Directorate of Rice Research, Hyderabad and Rice Research Station, Karjat, India. The seed material was grown in a glass house for 3 weeks and aerial tissue was harvested.

Table 1 Indian elite rice cultivars used in the present study

Name	Cross combination	Year of release	Days to 50% flowering	Grain type	Eco-system
1.Karjat-2	RPW-6-17/RP-4-14	1994	105	LS	IRM
2.Sugandha	Prabhavati/IET8573	1994	85	LS	--
3.Jal Lahari	Pankaj/Mahsuri/TKM6	1993	150	MS	SDW
4.Jalnidhi	Selection of Goanth	1993	150	LB	DW
5.Jal Priya	Select IET4060/Jalmanga	1993	130	LS	DW
6.Varsha	IR50/Mahsuri	1993	90	MS	IRE
7.Narmada	T(N)1/Basmati370	1991	110	LS	IRM
8.Vibhava	CR44-35/W1270B	1989	105	LS	IRM
9.Chandan	Sona/Manoharsali	1989	100	LS	IRM
10.Salivahana	RP5-32/Pankaj	1988	128	SB	RSL
11.Pothanna	IR579/WGL12708	1988	95	LS	IRE
12.Lalat	OBS677/IR2071/Vikram/W1263	1988	95	LS	IRM
13.Annada	Kumar/CR57-49	1988	85	MS	IRM

14.Indrayani	Ambemohr-157/IR8	1987	102	LS	IRM
15.Prasanna	IRAT8/N22	1986	65	LS	RUR
16.Rambha	Pankaj/W-1263	1985	120	MB	SDW
17.Gouri	T-90/IR-8/Vikram	1984	105	MS	IRM
18.Daya	Kumar/CR57-49	1984	105	MS	IRM
19.Sankar	Parijat/IET3225	1983	55	LB	RUR
20.Mandyavani	Cr1014/IR8(Sec.selection)	1982	100	MS	IRM
21.Subhadra	T(N)1/SR26B	1980	60	LB	RUR
22.Phalguna	IR-8/Siam29	1977	115	LS	RSL
23.Prakash	T90/IR8	1977	105	LS	IRM
24.Rasi	T(N)1/Co-29	1977	85	MS	IRE
25.Akaash	IR8/N-22	1977	80	SB	RUR
26.Bhavani	Peta/BPI76	1976	105	LS	IRM
27.Surekha	IR8/Siam29	1976	104	LS	IRM
28.Parijat	TKM6/T(N)1	1976	70	MS	RUR
29.Intan	Introd. from Indonesia	1975	135	LS	RSL
30.Mangala	Jaya/S317	1975	80	MS	IRE
31.Hema	T-141/IR-8-246	1974	105	LB	IRM
32.Rajeshwari	T-19/IR-8	1974	100	SB	IRM
33.Vani	IR8/CR1014	1973	105	LS	IRM
34.Tellahamsa	HR12/T(N)1	1971	90	MS	IRE
35.Jal Manga	Sel. from Badhan	1969	130	SB	SDW
36.Madhukar	Self from Gonda	1969	120	MS	SDW
37.Jaya	T(N)1/T141	1968	100	LB	IRM
38.Karjat-1	Halmaldiga/IR36	--	110	--	--
39.Karjat-18	---	--	--	--	--
40.Karjat-23	---	--	--	--	--
41.Karjat-35-3	HR-19/IR8	--	90	--	--
42.Karjat-184	T(N)1/Kolamba540	--	100	--	--
43.Ratnagiri-1	IR8/RTN24	--	110	--	--
44.Ratnagiri-2	RTN-60-8-1/Warangal	--	140	--	--

NB : IRE (Irrigated Rice Early duration), IRM (Irrigated Rice Medium duration), RUR (Rainfed Upland Rice), RSL (Rainfed Shallow Water or Low Land), SDW (Semi Deep Water) and DW (Deep Water).

DNA extraction and RAPD analysis : DNA was extracted from young leaves using the hexadecyltrimethyl ammonium bromide (CTAB) method described by Rogers and Bendich (1988). RAPD analysis was carried out on MJ Research Thermocycler with modified protocol of Williams *et al* (1990). The reaction consisted of 20ng of template DNA, 100μM of each dNTP (USB,

Amersham), 10pmoles of operon primer (Operon Technologies, Inc., Alameda,CA), 0.3U Taq Polymerase (Biogenei), and 1 x assay buffer (10 mM Tris, pH8.3, 50mM KCl, 1.5mM $MgCl_2$) in 25μl volume. The thermocycler was programmed for initial denaturation at $94^{0}C$ for 5min followed by 45 cycles of $94^{0}C$ (1min), $36^{0}C$ (1min), and $72^{0}C$ (2min) with a final extension at $72^{0}C$ for 5min. Amplified DNA samples were electrophoresed on 1.4% agarose gels in 1XTAE buffer at 40 to 60V for 3 to 4 h, stained with EtBr and photographed under UV light.

Statistical analysis : Evaluation of fragment patterns and statistical calculations were performed according to Wetton *et al.* (1987). A Similarity coefficient (F) between two DNA fingerprints was calculated as

$F = 2N_{AB}/(N_A+N_B)$

where N_{AB} was the number of bands shared by fingerprints A and B, and N_A and N_B were the total number of bands present in fingerprints A and B, respectively. The chance occurrence that two different genotypes would exhibit identical fragment profiles was then calculated as the average F raised to mean number of fragments per genotype. The dendrogram was constructed using UPGMA (unweighed pair-group method with arithmetic averages) with the computer program Taxan 4.0.

RESULTS

Initially, four elite rice cultivars ('Rasi', 'Madhukar', 'Karjat-1' and 'Ratnagiri-2') were subjected to RAPD analysis using 40 primers (OPA1 to 20 and OPB1 to 20). Nine primers (OPA5 and 6; OPB 1,2,3,4,15,19 and 20) gave no DNA amplification, eight primers (OPA 1,2,13,14,16,19and 20; OPB16) resulted into faint bands and two primers (OPB6 and 9) gave monomorphic banding patterns. Of the remaining primers, eight primers (Table 1) gave reproducible and polymorphic patterns and were used for further evaluation of all 44 elite rice cultivars.

Using eight primers ,a total of 40 reproducible fragments per genotype were amplified, 80% of which showed polymorphism. Figure 1 and Figure 2 demonstrate the RAPD pattern of 44 Indian elite rice cultivars using primer OPA 13 and OPB 18, respectively. The RAPD pattern generated with primer OPA13 was highly polymorphic and contained maximum number of bands (Fig 1a and 1b). The cultivar specific amplicons obtained with OPA13 have been indicated by arrows. OPA7 and OPA17 also yielded cultivar specific bands for 'Rambha', 'Gouri', 'Sugandha', 'Subhadra', 'Mangala', 'Jal Priya' and 'Jal Manga'.

Table 2 Operon primers used in the RAPD assay

Primer no.	Sequence 5' to 3'	No. of amplified bands
1.OPA 1	CAGGCCCTTC	4
2.OPA7	GAAACGGGTG	2
3.OPA12	TCGGCGATAG	3
4.OPA13	CAGCACCCAC	13
5.OPA17	GACCGCTTGT	6
6.OPA18	AGGTGACCGT	6
7.OPB7	GGTGACGCAG	4
8.OPB18	CCACAGCAGT	2

Based on the RAPD data, cluster analysis using genetic distance values was performed to generate a dendrogram showing genetic relationships within Indian elite rice cultivars (Fig 3). Forty four elite rice cultivars grouped into two big clusters consisting of 25 and 17 cultivars, respectively, while 'Bhavani' and 'Jalmanga' joined the two clusters distantly. The highest similarity index value (0.90) was found between the cultivars 'Ratnagiri-2' and 'Salivahana' and also between 'Prakash' and 'Vani' while the least similarity was found between 'Hema' and 'Bhavani'.

Fig 1a and 1b: Pattern of RAPD fragments generated with primer OPA13 for the 44 Indian elite rice cultivars. M : 1Kb ladder Lane no 1 : Hema, 2 : Tellahamsa, 3 : Rambha, 4 : Jal Lahari, 5 : Phalguna, 6 : Intan, 7 : Rajeshwari, 8 : Jaya, 9 : Indrayani, 10 : Bhavani, 11 : Rasi,12 : Lalat, 13 : Prakash, 14 : Gouri, 15 : Sankar, 16 : Annada, 17 : Akaash, 18 : Sugandha, 19 : Madhukar as a positive control, 20 : Subhadra, 21 : Vibhava, 22 : Mangala, 23 : Karjat-1 as a positive control, 24 : Ratnagiri-2 as a positive control, 25 : Varsha, 26 : Jal Priya, 27 : Pothanna, 28 : Jalnidhi, 29 : Salivahana, 30 : Surekha, 31 : Daya, 32 : Narmada, 33 : Parijat, 34 : Jal Manga, 35 : Vani, 36 : Mandhyavani, 37 : Chandan, 38 : Prasanna, 39 : Karjat-1, 40 : Karjat-2, 41 : Karjat-18, 42 : Karjat-23, 43 : Karjat-35-3, 44 : Karjat-184, 45 : Ratnagiri-1, 46 : Rantagiri-2 and 47 : Madhukar. Lanes 1,8,12,26 and 27 show poor amplification band pattern.

Fig. 1A

Fig. 1B

31
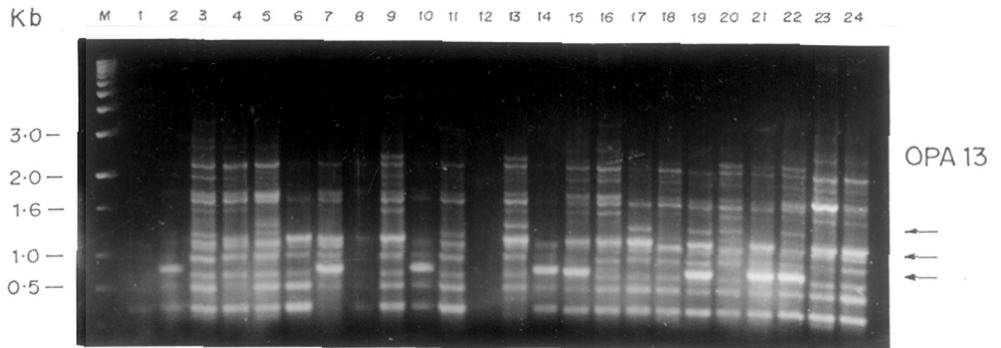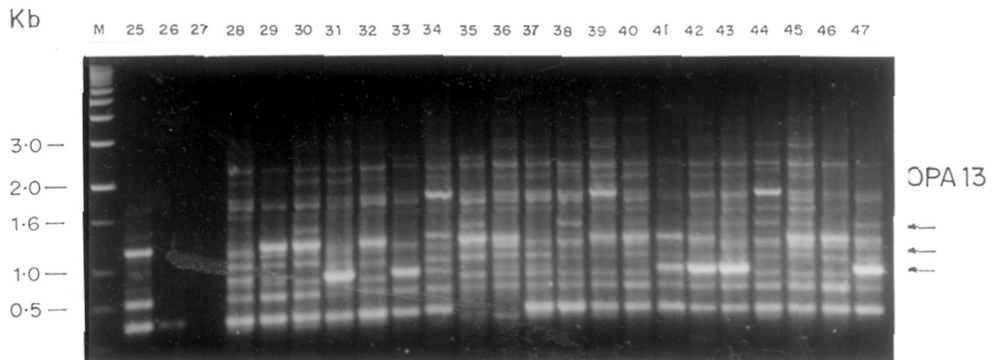
Fig 2a and 2b: Pattern of RAPD fragments generated with primer OPB18 for the 44 Indian elite rice cultivars. M : φ X 174 digested with *Hae*III. Lane numbers 1-47 are same as figure 1a and 1b.
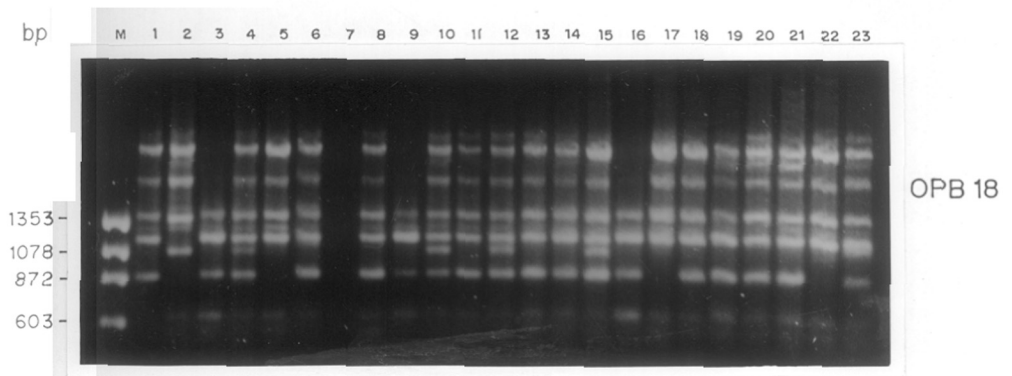
Fig. 2A

Fig. 2B

Figure 3 : Dendrogram showing genetic relationships among 44 Indian elite rice cultivars

Genetic similarity

| 0.90 | 0.88 | 0.81 | 0.77 | 0.73 | 0.68 | 0.64 | 0.60 | 0.55 | 0.51 | 0.46 |

A1
HEMA
INTAN
AKAASH
POTHANNA
TELLAHAMSA
JAL PRIYA

A2
RAMBHA
RATNAGIRI-2
SALIVAHANA
NARMADA
PRASANNA

PHALGUNA
JAYA

A3
RASI
DAYA
CHANDAN
KARJAT-2
RATNAGIRI-1
SUGANDHA
JALNIDHI

SUREKHA
MANDYAVANI
KARJAT-184
LALAT
VARSHA

B1
JAL LAHARI
PRAKASH
VANI
ANNADA

B2
RAJESWARI
GOURI
MADHUKAR

B3
INDRAYANI
SANKAR
SUBHADRA
KARJAT-1

B4
VIBHAVA
MANGALA
PARIJAT

B5
KARJAT-18
KARJAT-23
KARJAT-35-3

BHAVANI
JAL MANGA

DISCUSSION

In the present work, we have used RAPD markers to establish relationships among 44 Indian elite rice cultivars. This is the first attempt to characterize elite varieties of rice that are commonly used in India for breeding program. Of the 44 cultivars used, the present genetic similarity data has grouped 42 cultivars into two big clusters(A&B). The first cluster (A) containing three major subgroups has 25 elite lines with a similarity range of 0.64 to 0.90. In the first subgroup A_1, three cultivars viz 'Hema', 'Akaash' and 'Pothanna' have a common IR background. 'Intan', the elite line from Indonesia is also present in this group. The remaining two subgroups of this cluster A_2 and A_3 do not show any common parent in the pedigree analysis except 'Rambha' and 'Salivahana' belonging to subgroup A_2, which have 'Pankaj' as the common parent. The second cluster has 5 small subgroups viz B_1 to B_5. The subgroup B_2 showing 78% similarity has 'IR8' as the common parent while the subgroup B_4 sharing common parent 'T(N)1',has 71% similarity. These results indicate that at the smaller subgroup level, the elite rice cultivars are genetically more similar. Our data and pedigree analysis reveal that genetic diversity of some elite rice cultivars has been reduced due to intensive breeding efforts.

The semi-dwarf irrigated rice, 'IR8', introduced with the green revolution is responsible for narrowing the genetic base in irrigated rice (Hargrove 1979). 'IR8', 'T(N)1', 'TKM6' and 'IET (Kasturi)' have been used most frequently in the breeding program to develop the elite rice varieties used in the present study. Analysis of the pedigree of the crosses suggests that eventhough many elite varieties have been produced, only a limited genetic variability is available within these cultivars. 'IR8' was used as a parent in 43% of the varieties used in this study. The elite rice varieties developed, therefore, represent only a narrow genetic base. The viable alternative to increase the genetic variability as suggested by several authors (Hull 1945; Penny *et al* 1967; Fehr 1987) is loosening the selection criteria by including early segregating lines and by recurrent selection methods. In

addition, the genetic diversity of wild genotypes can also be exploited using the DNA markers developed so far (Tanksley *et al* 1989).

Eventhough, RAPDs indicate the presence of a narrow genetic base in the genotypes studied, all the 44 Indian elite rice varieties represent the extensive genetic diversity for various morphological, biological and physical characters. 'IR8' and 'T(N)1' have been used in the early phase of rice improvement for generating dwarf varieties. Subsequently, Indian germplasm has been extensively used in developing rice varieties resistant to a few biotic and abiotic stresses. Various donors of different traits have been used for developing different elite varieties as shown in Table 3. The cultivars showing common traits (Table 3) do not necessarily cluster together with RAPD markers (Fig. 3). 'Phalguna', 'Surekha', 'Pothanna' which are gall midge resistant are scattered among different subgroups of cluster 'A'. 'Bhavani' and 'Jal Manga' which are deepwater and irrigated cluster together. 'Intan' grown

Table 3 Donors used for developing different traits in the present elite germplasm

Character	Variety
1. Dwarfism and N response	TN1, IR8 (DGWG gene Taiwan Dwarf) is associated with non - lodging and N responsive varieties
2. Grain quality	T90, T141, HR19, GEB 24, Mahsuri, Bas-370, Ambemohr
3. Insect/disease resistance	GM : W1263, Siam 29 BPH and GLH : PTB2, PTB 21, PTB 33 Blast : Co 29, Co 4 RTV : Latisail, Ambemohr 159, Katasibhog
4. Uplands	N22, Finegora
5. Deep water	Jalmanga.

in western ghats; 'Akash', 'Pothanna' and 'Tellahamsa' irrigated cultivars of Andhra Pradesh and 'Jal Priya', a deepwater rice of Bihar and Uttar Pradesh all cluster together. Usually, the donors used were for specific traits as mentioned in Table 3 and selections were done in order to identify a genotype containing a particular trait alongwith other elite characters. Hence, the clusters cannot specifically classify the genotypes based on pedigree.

Analysis of plant DNA using recent techniques has provided DNA markers that can be used to demonstrate the distinctiveness, uniformity and stability of a plant variety (Morell *et al* 1995). Apart from genetic evaluation of elite rice cultivars, RAPDs as well as mini- and microsatellite DNA markers have the potential for plant varietal identification. The cultivar specific patterns can be further evaluated to develop locus specific tags for identification of varieties. These DNA markers will become the markers of choice for certifying newly released varieties thus protecting plant breeder's rights.

In conclusion, the present DNA marker analysis has revealed the occurrence of a narrow genetic base in elite rice varieties in India. It also gives a suggestion to rice breeders to broaden the genetic base by various procedures including introgression of genes from wild germplasm.

CHAPTER II

SECTION - B

USE OF DNA MARKERS IN PREDICTION OF

HYBRID PERFORMANCE AND HETEROSIS IN

RICE

INTRODUCTION

Exploitation of heterosis in F_1 hybrids, produced by crossing genetically divergent pure lines, is a well recognized breeding practice to increase yield in rice. However, the generation of a large number of crosses under field conditions for the selection of a superior hybrid is extremely tedious and time consuming. Hence an alternative method which would help in prediction of hybrid performance by analyzing the parental lines is highly desirable. The level of genetic diversity between two parents has been proposed as a possible predictor of F_1 performance. Mahalanobis (1936) D2 statistic and Griffings' (1956) combining ability analysis have been extensively used in predicting the hybrid performance on the basis of morphological traits. However, these methods require extensive field testing and crossing and hence, a need is now felt for finding out genetic distances at the molecular level and predicting the hybrid performance. The results obtained using isozyme data (Li *et al.* 1982; Deng and Wang 1984) have revealed that distances computed from isozyme analysis are in some cases significantly correlated to heterosis, but the correlations are generally too low for the distances to be of practical predictive value (Peng *et al.*, 1988, 1991).

The advent of DNA marker technology has led to a considerable interest in predicting hybrid performance in crop breeding programs. If the DNA markers could be used to predict heterosis prior to expensive field testing, much of the field work associated with making crosses and field evaluation would be eliminated and hybrid breeding programs would be accelerated. Initially RFLP's were used in several crops and prediction of hybrid performance was shown to be highly dependent on the germplasm involved in the study (Lee *et al.* 1989; Godshalk *et al.* 1990; Melchinger *et al.* 1990a, b; Dudley *et al.* 1991; Melchinger *et al.* 1992; Boppenmeirer *et al.* 1992; Zhang *et al.* 1994, 1995, 1996). Some attempts showed that the correlation between genetic distances and hybrid values would increase within a closely related germplasm used for such analysis (Frei *et al.* 1986; Stuber *et al.* 1992). Melchinger *et al.*(1992) concluded that RFLP distances

would not be useful for predicting the value of crosses between lines belonging to different heterotic groups and this was further supported by Boppenmeier *et al.* (1992). In our own research work on pearl millet, we have reported that genetic distances based on (GATA)$_4$ microsatellite and RAPDs might be useful for grouping of parents but not for predicting heterotic combinations (Chowdari *et al.* 1998a; 1998b). Xiao *et al.* (1996b) indicated that genetic distances based on RAPD's and SSRs might be useful for predicting yield potential and heterosis of intra-subspecific hybrids, but not inter-subspecific hybrids in rice. Reports of Zhang *et al.* (1994, 1995, 1996) clearly showed increased correlation between heterosis and marker heterozygosity with increasing relatedness in the rice germplasm studied suggesting usefulness of DNA markers in cluster analysis and for prediction of hybrid performance in rice.

Systematic evaluation of hybrids in rice for commercial heterosis has been initiated in 1989 and only a few hybrids performing comparatively well at more than one location have been obtained so far (Virmani 1996). This location-specific performance appears to be dependent on the types of parental lines involved. Several efforts have been made to develop high yielding and region specific hybrids in India. Presently, ongoing programs are aimed at developing stable CMS lines with various nuclear background to suit different agroclimatic regions. However so far, there have been no reports of using DNA markers to analyze the CMS and restorer lines for identification and developing heterotic parents and hybrids in rice breeding program. In the present study, we have first investigated genetic diversity in Wild Abortive (WA) type cytoplasmic male sterile lines and their compatible restorer lines using RAPD-PCR approach. Next an attempt has been made to correlate the genetic distances of the genotypes with hybrid performance and heterosis for prediction of hybrid performance and heterosis with DNA markers.

MATERIALS AND METHODS

Plant Materials and field experiments : Two CMS lines (IR 58025A and IR62829A) and 16 restorers lines (RTN 1, RTN 2, RTN 3, RTN 24, RTN 68, RTN 73, RTN 711, PNL 1, PNL 2 KJT 1, KJT 2, KJT 3, KJT 4, KJT 14-7, KJT 184 and PLG 1) of rice were used in the present study. The crossing of two CMS lines with 16 restorers producing 32 F_1 hybrids was carried out at Dapoli, Maharashtra. The hybrids were grown in a Randomized Block Design with three replications and the data were recorded on 16 characters viz., plant height, tillers per plant, productive tillers, percentage of productive tillers, panicle length, grains per panicle, unfilled grains per panicle, percentage grains fertility, flag leaf initiation, days to 50% flowering, days to maturity, biological yield, dry matter, harvest index, 100-grain weight and grain yield per plant. Heterosis for each cross was computed as a deviation from the mid-parent and better parent values.

DNA markers and data analysis : DNA was extracted from young leaves using the hexadecyltrimethyl ammonium bromide (CTAB) method described by Rogers and Bendich (1988). RAPD analysis was carried out with 46 operon primers of OPA, OPB, OPC, OPD and OPJ series. PCR reactions were performed in volumes of 25 µl consisting of 100µM each of dATP, dTTP, dCTP and dGTP, 5pmoles of primer, 20ng of genomic DNA, 1 x reaction buffer and 0.5 Units of Taq DNA Polymerase. Samples were amplified in a Perkin Elmer Cetus 48-well DNA thermal cycler programmed with a step of 3 minutes at 94^0C for initial denaturation. This was followed by 45 cycles of 1 minute at 94^0C, 1 minute at 36^0C and 2 minutes at 72^0C. The final cycle was followed by a 5 minutes final extension step at 72^0C. Amplified products were separated on a 1.4% agarose gel in 1xTAE buffer and were visualized by ethidium bromide staining. Band profiles for each parent were given as 1 for the presence or 0 for the absence of that band. Genetic similarities (GS) were calculated for all pairwise combinations of parents using methods described by Nei (1987). Correlations were calculated between F1 performance, heterosis over midparent, better parent and the genetic distance.

RESULTS

DNA polymorphism and cluster analysis in rice

A total of 18 rice genotypes including two CMS lines were subjected to RAPD-PCR analysis to detect DNA polymorphism. Out of 46 operon primers attempted, 35 primers gave polymorphic patterns and hence were informative. These primers generated 186 amplified products, in which 49 were monomorphic. Among the 35 primers, OPB15 and OPB17 amplified maximum number of bands (13 and 11, respectively) with maximum number of polymorphic (9 each) bands. Figure 1 and 2 includes the DNA amplification patterns of all the 18 rice genotypes under consideration with OPA11, OPA17, OPB15 and OPB17. As seen in these figures all the genotypes could be distinguished but no location or region specific markers for restorers were obtained.

Similarity index values for RAPD patterns were calculated for all the possible pairwise comparisons which ranged from 0.86 (RTN 73 and PNL 2; KJT 2 and PNL1) to 0.61 (IR 62829 A and RTN 24) (Table 1). Based on the RAPD data, cluster analysis was performed and the dendrogram generated 4 clusters, in which a CMS line IR 58025 A was closer to two clusters including restorer lines RTN 1, RTN 73, PNL 2, RTN 711 and KJT 184 in one cluster and KJT 2, PNL 1 and KJT 3 in another cluster, whereas another CMS line IR 62829 A clustered with restorer lines KJT 1 and PLG 1 only. Remaining restorer lines, RTN 2, KJT 14-7, RTN 68 and KJT 4 formed one cluster while RTN 24 and RTN 3 which were out grouped were not closer to either of the CMS lines (Figure 3).

Hybrid performance and heterosis

Thirty two hybrids were evaluated for yield and its component characters described in materials and methods. The hybrid performance and heterosis of the 32 F_1 hybrids are shown in Table 2. The degree of heterosis varied considerably from trait to trait. The unfilled grains per panicle exhibited highest mid parental heterosis followed by tillers per plant and productive tillers per plant, while unfilled grains per panicle also showed maximum

Figure 1A and 1B : RAPD patterns of rice CMS and restorer lines with OPA11 and OPA17. M : φ x 174 / *Hae*III DNA marker Lanes 1 to 18 are IR 58025 A (1), IR 62829 A (2), RTN 1 (3), RTN 2 (4), RTN 3 (5), RTN 24 (6), RTN 68 (7), RTN 73 (8), RTN 711 (9), KJT 1 (10), KJT 2 (11), KJT 4 (12), KJT 184 (13), PNL 1 (14), PNL 2 (15), KJT 3 (16), KJT 14-7 (17) and PLG 1 (18).
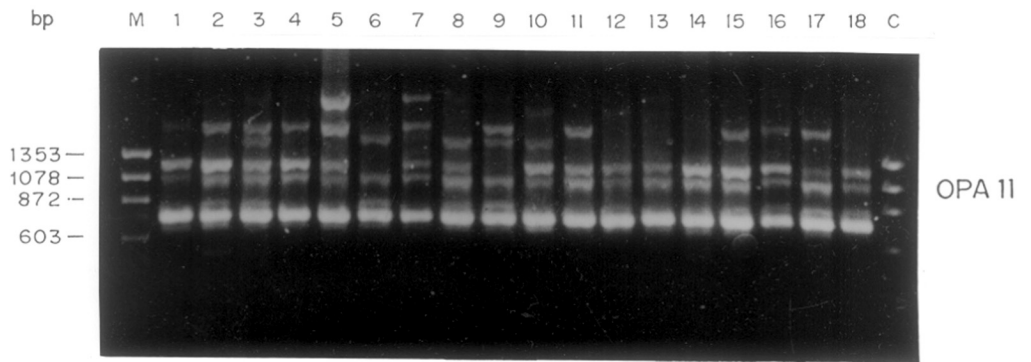
Fig. 1A
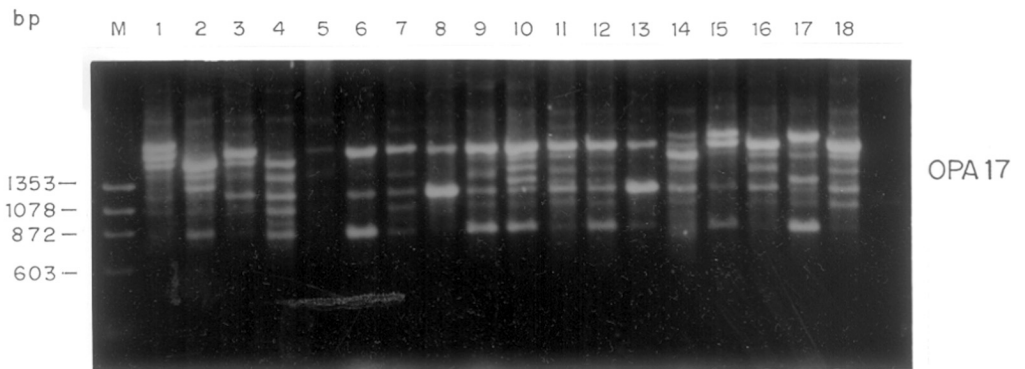
Fig. 1B

Figure 2A and 2B : RAPD patterns of rice CMS and restorer lines with OPB15 and OPB17. M : ϕ x 174 / *Hae*III DNA marker Lanes 1 to 18 are IR 58025 A (1), IR 62829 A (2), RTN 1 (3), RTN 2 (4), RTN 3 (5), RTN 24 (6), RTN 68 (7), RTN 73 (8), RTN 711 (9), KJT 1 (10), KJT 2 (11), KJT 4 (12), KJT 184 (13), PNL 1 (14), PNL 2 (15), KJT 3 (16), KJT 14-7 (17) and PLG 1 (18).
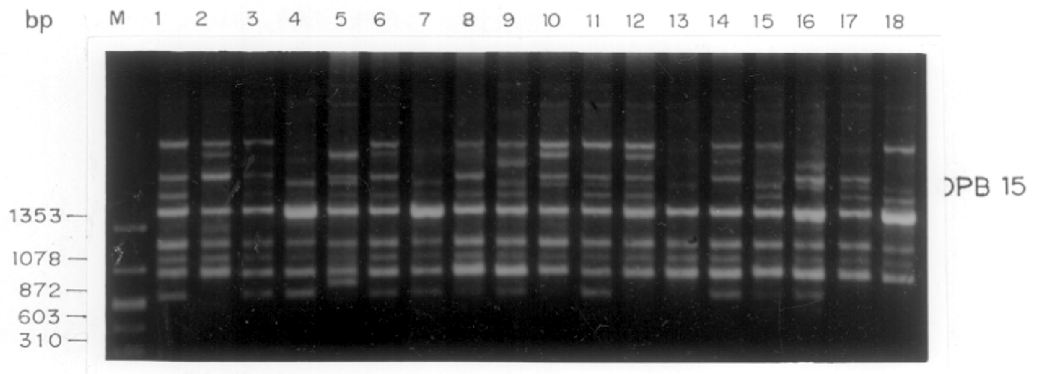
Fig. 2A
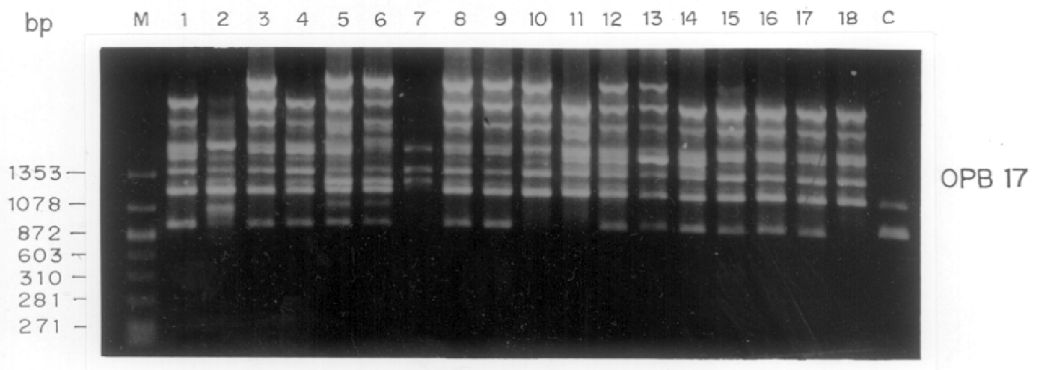
Fig. 2B

Figure 3   Dendrogram showing genetic relationships among rice CMS and restorer lines used in the present study
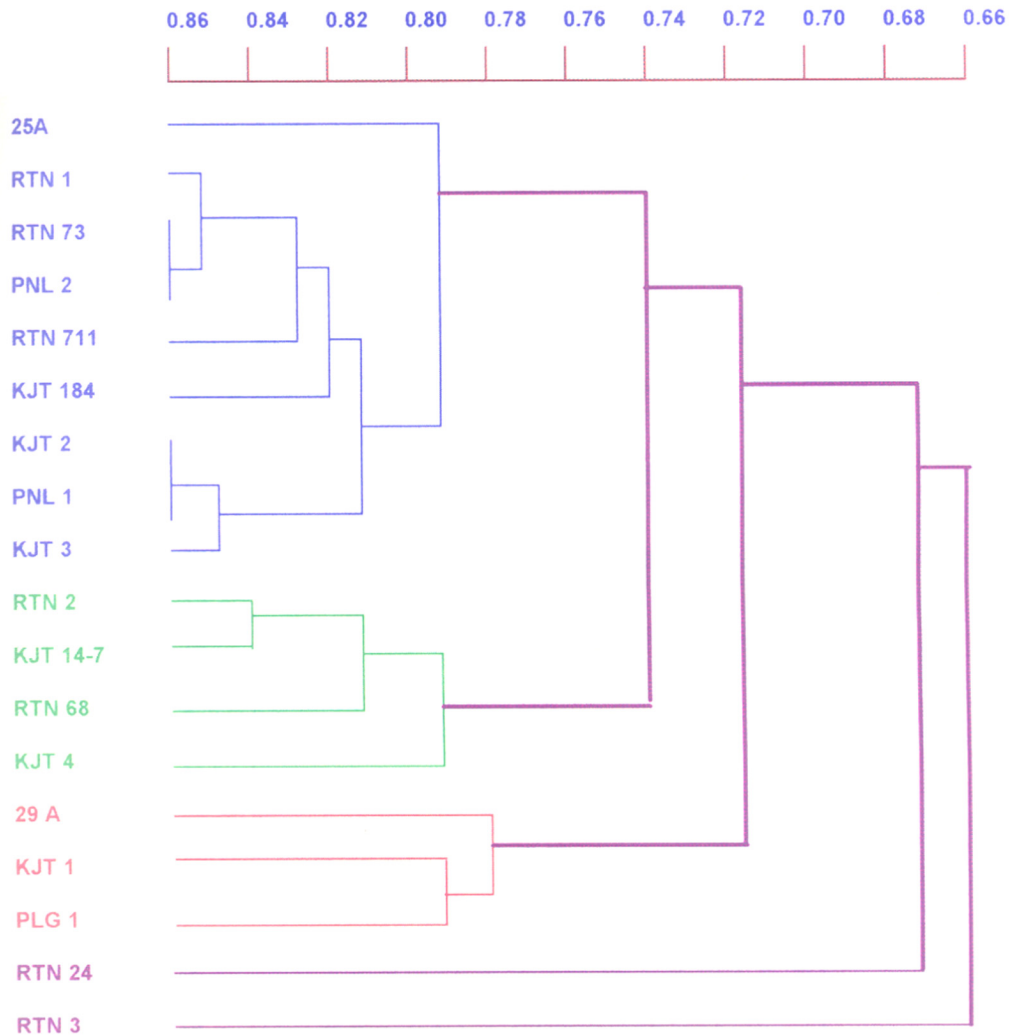
Table 1 The genetic similarity matrix among 18 rice genotypes used in the present study

	25A	RTN 1	RTN 73	PNL 2	RTN 711	KJT 184	KJT 2	PNL 1	KJT 3	RTN 2	KJT 14-7	RTN 68	KJT 4	29 A	KJT 1	PLG 1	RTN 24	RTN 3
25A	1.00																	
RTN 1	0.79	1.00																
RTN 73	0.79	0.85	1.00															
PNL 2	0.78	0.81	0.86	1.00														
RTN 711	0.74	0.83	0.81	0.78	1.00													
KJT 184	0.78	0.82	0.83	0.84	0.81	1.00												
KJT 2	0.81	0.82	0.81	0.81	0.77	0.79	1.00											
PNL 1	0.80	0.82	0.82	0.85	0.74	0.81	0.86	1.00										
KJT 3	0.79	0.84	0.80	0.79	0.77	0.80	0.85	0.82	1.00									
RTN 2	0.74	0.78	0.78	0.78	0.77	0.78	0.80	0.77	0.83	1.00								
KJT 14-7	0.80	0.81	0.80	0.82	0.79	0.83	0.81	0.82	0.85	0.84	1.00							
RTN 68	0.75	0.77	0.79	0.78	0.75	0.75	0.81	0.78	0.78	0.82	0.81	1.00						
KJT 4	0.71	0.75	0.80	0.75	0.74	0.77	0.78	0.75	0.75	0.79	0.79	0.71	1.00					
29 A	0.72	0.69	0.67	0.69	0.68	0.69	0.72	0.71	0.71	0.70	0.74	0.68	0.69	1.00				
KJT 1	0.73	0.75	0.78	0.75	0.74	0.78	0.78	0.77	0.75	0.73	0.78	0.74	0.77	0.78	1.00			
PLG 1	0.73	0.73	0.75	0.75	0.71	0.76	0.78	0.78	0.78	0.74	0.78	0.71	0.75	0.72	0.80	1.00		
RTN 24	0.67	0.71	0.78	0.74	0.78	0.73	0.71	0.69	0.69	0.71	0.70	0.73	0.71	0.61	0.70	0.63	1.00	
RTN 3	0.66	0.70	0.75	0.71	0.71	0.71	0.73	0.71	0.71	0.73	0.74	0.74	0.72	0.71	0.73	0.75	0.66	1.00

Table 2 Mean and range of hybrid performance and heterosis in the 32 hybrids

Trait	HP Mean	HP Range	HMP(%) Mean	HMP(%) Range	HBP Mean	HBP Range
1) Plant height (cm)	71.55	59.33 - 87.16	10.59	-10.57 - 30.24	31.16	-3.15 - 66.85
2) Tillers per plant	11.90	6.93 - 17.93	31.30	-40.05 - 121.85	12.22	-55.17 - 97.07
3) Productive tillers per plant	10.09	6.1 - 16.6	23.55	-26.10 - 124.32	1.16	-44.79 - 90.80
4) % of productive tillers per plant	84.85	63.83 - 100.00	-4.60	-19.12 - 21.45	-9.94	-26.68 - 19.95
5) Panicle length (cm)	23.71	15.00 - 29.03	22.81	-7.85 - 50.97	3.11	-30.01 - 32.68
6) Grains per panicle	158.19	77.53 - 282.40	0.74	-46.72 - 68.90	-12.32	-57.25 - 54.75
7) Unfilled grains per panicle	42.01	15.20 - 117.90	90.70	-31.01 - 346.71	145.61	1.33 - 540.40
8) % of grain fertility	73.81	48.71 - 88.69	-13.94	-43.90 - 1.45	-18.51	-90.60 - 0.40
9) Flag leaf initiation	67.96	55.00 - 77.33	-5.78	-25.54 - 20.20	4.12	-12.24 - 23.33
10) Days to 50% flowering	81.97	75.66 - 90.33	2.99	-14.80 - 21.94	12.75	0.91 - 27.53
11) Days to maturity	112.11	102.00 - 125.00	0.18	-14.74 - 17.08	6.42	-7.50 - 23.95
12) Biological yield (g)	74.88	42.43 - 163.96	-0.45	-48.04 - 90.99	-50.84	-54.98 - 161.91
13 Dry matter (g)	56.29	27.00 - 110.00	-3.81	-55.73 - 87.35	-21.21	-65.38 - 55.66
14) Harvest index (%)	24.66	1.70 - 37.37	6.60	-63.99 - 68.70	-17.42	-69.72 - 44.51
15) 100 - grain weight (g)	2.22	5.46 - 2.80	4.65	-19.08 - 58.49	16.51	-111.20 - 100.00
16) Grain yield per plant (g)	18.26	4.60 - 53.96	9.25	-65.91 - 162.39	-9.44	-73.63 - 98.76

HP - Hybrid performance

HMP - Heterosis over midparent

HBP - Heterosis over better parent

heterosis over better parent followed by plant height and 100-grain weight. Unfilled grains per panicle (-31.01% to 346.71%) showed maximum difference between highest and lowest values followed by yield potential (65.91% to 162.39%) and other important yield contributing characters viz., tillers per plant (-40.05% to 121.85%) and productive tillers per plant (-26.10% to 124.32%). Considering the better parent heterosis, maximum differences in heterotic effects were observed for unfilled grains per panicle (1.33% to 540.40%) followed by 100-grain weight (-111.2% to 100.0%), yield potential (-73.63% to 98.76%) and biological yield (-54.98% to 161.91%).

Relationship of genetic distance to hybrid performance and heterosis

The correlations of genetic distance with the hybrid performance and heterosis of the 32 F_1 hybrids were estimated by regressing heterosis or trait values on the genetic distance (Table 3). It is evident from the Table 3 that the genetic distance was negatively correlated with hybrid performance , heterosis over midparent and better parent for plant height, percentage of productive tillers and grain yield per plant. Many traits showed no association with genetic distance. The maximum negative correlation of genetic distance with hybrid performance was observed with percentage of productive tillers followed by panicle length, plant height and grains per panicle, while only tillers per plant showed positive association. Maximum negative association between genetic distance and mid parental heterosis was observed in percentage of productive tillers followed by plant height., biological yield and grain yield per plant. By and large, a similar trend was also observed for association between genetic distance and better parent heterosis.

Discussion

The DNA markers involving RAPDs have revealed their potential in distinguishing 18 rice parental lines and clustering them into four groups. It is for the first time that CMS lines and restorer lines which are used in hybrid rice program are investigated employing DNA markers. The latter thus provide

Table 3 Correlations of genetic distance with hybrid performance and heterosis over midparent and better parent

Characters	HP			HMP			HBP		
	Total	25A	29A	Total	25A	29A	Total	25A	29A
1) Plant height	-0.30	-0.02	-0.03	-0.48**	-0.13	-0.14	-0.40*	0.03	0.12
2) Tillers per plant	0.17	-0.14	-0.32	0.08	-0.15	-0.50**	-0.01	-0.15	-0.51**
3) Productive tillers per plant	-0.07	-0.34	-0.54**	-0.11	-0.39*	-0.60**	-0.16	-0.36	-0.59**
4) % of productive tillers per plant	-0.45**	-0.22	-0.50**	-0.53**	-0.32	-0.53**	-0.46**	-0.28	-0.48**
5) Panicle length	-0.31	0.08	0.02	0.15	-0.07	0.02	-0.38*	-0.16	0.01
6) Grains per panicle	-0.30	0.10	-0.00	-0.27	-0.14	-0.19	-0.16	0.01	-0.22
7) Unfilled grains per panicle	-0.11	0.19	0.00	-0.05	0.14	-0.27	0.05	0.23	-0.03
8) % of grain fertility	-0.02	-0.23	0.12	-0.05	-0.23	0.20	-0.22	-0.61**	0.14
9) Flag leaf initiation	-0.16	0.24	-0.00	0.02	-0.02	0.24	0.09	0.06	-0.04
10) Days to 50% flowering	-0.25	0.04	-0.07	0.01	-0.16	0.22	0.24	-0.16	0.32
11) Days to maturity	-0.12	-0.12	0.14	-0.00	-0.25	0.21	-0.05	-0.14	0.02
12) Biological yield	-0.23	-0.25	-0.18	-0.33*	-0.34*	-0.27	-0.32	-0.26	-0.18
13) Dry matter	-0.22	-0.28	-0.16	-0.26	-0.33*	-0.17	-0.29	-0.28	-0.17
14) Harvest index	-0.05	0.18	0.00	-0.11	0.14	-0.02	-0.10	0.11	-0.04
15) 100 - grain weight	-0.20	-0.46**	0.18	0.11	0.11	0.32	0.11	0.42**	0.11
16) Grain yield per plant	-0.21	-0.14	-0.09	-0.32	-0.26	-0.19	-0.31	-0.43**	-0.13

HP - Hybrid performance HMP - Heterosis over midparent HBP - Heterosis over better parent

*P = 0.05   **P = 0.01

an alternative approach to Mahalanobis' (1935) arduous D^2 analysis based on morphological traits. However, comparative study of DNA polymorphism and clustering analysis and D^2 clustering pattern may be necessary for drawing valid conclusions.

Predictions of hybrid performance and heterosis can be carried out based on genetic distance or by studying diallel crosses. This is the first report where genetic distance in Indian rice genotypes is estimated from a DNA marker based approach and is correlated with hybrid performance and heterosis. Extensive studies have been carried out to explore the utility of DNA marker data in field studies of important crops such as rice, wheat, maize, brassica and oil see crops. Zhang *et al.* (1995), for example, have reported that the relationship between molecular marker heterozygosity and heterosis is variable, depending on the genetic materials and the diversity of rice germplasm because of the complexity of the genetic basis of heterosis. In rice, the inter subspecies (*Indica* and *Japonica*) crosses have shown no positive correlation with genetic distance and hybrid performance and heterosis (Xiao *et al.* 1996). In general, it is well accepted that the association of marker-based genetic distance with hybrid performance and heterosis depends on the type of crop and the germplasm under study. In the present work, the correlations of genetic distances with hybrid performance and heterosis were either negative or non-significant and very weak for many traits putting a question mark on their utility in prediction of hybrid performance. These findings are contrary to those reported by Xiao *et al.* (1996) for intra-specific hybrids.

The effect of IR 58025 A and IR 62829 A CMS lines in F_1 nuclear background has shown different significant levels of correlations for productive tillers and grain yield per plant (Table 3). Since heterosis is a phenomenon of superior growth, development, differentiation, and maturation caused by the interaction of genes and environment, a simple explanation of heterosis based solely on the nuclear genome heterozygosity appears untenable (Srivastava 1983). Chen *et al.* (1987) have demonstrated positive and negative effects of cytoplasm on agronomic as well as physiological traits.

They have observed that male sterility-inducing cytoplasms vary in their effects depending on the nuclear background of the hybrids and some of the effects are considered nucleo-cytoplasmic interactions, especially for yield, and panicle exertion. In our report, the correlations are mostly negative and significant for plant height, productive tillers, 100 grain weight and grain yield per plant. This is an important indication before using DNA marker technology for predictive purpose in hybrid breeding programs. The genetic diversity measures based on DNA markers that have not been associated with QTL for yield or related traits will be having equal chances of prediction either positive or negative. The genomic diversity studies with more number of probes may circumvent this problem to some extent.

In summary, the results from the current study have shown that parental lines could be identified and grouped into different clusters based on DNA marker technology providing an alternative for Mahalanobis' D^2 analysis. The data, however, could not generate significant predictive values for hybrid performance and heterosis, an important indication before using DNA marker technology for predictive purpose in hybrid rice breeding programs.

CHAPTER III

PEARL MILLET

In the present section, I have studied the genetic diversity of cultivars and landraces using micro and minisatellites and RAPD markers. It is essential to identify suitable DNA fingerprinting markers for genotype identification and assessment of genetic relationship. Further, I have made an attempt to correlate genetic distance values for the prediction of heterotic combinations in hybrid program. It is for the first time that such a study has been carried out on pearl millet in India and it will have relevance in developing superior varieties of pearl millet. The work on landraces of pearl millet is especially very important while introgressing desired traits from landraces to cultivars.

CHAPTER III

SECTION - A

GENOTYPE IDENTIFICATION AND ASSESSMENT OF GENETIC RELATIONSHIPS IN PEARL MILLET [*PENNISETUM GLAUCUM* (L.) R.BR] USING MICROSATELLITES AND RAPDS

INTRODUCTION

Importance of pearl millet and its limitations

Pearl millet is the fourth most important food crop of the world next to rice, wheat and sorghum. It is the most drought tolerant of all the domesticated cereals and is grown in regions with 200-600 mm of annual rainfall where no other cereal crop can be grown. The nutritive value / content of pearl millet is higher than rice and wheat (Uprety and Austin 1972). Soon after its domestication, pearl millet became widely distributed across the arid, semiarid, and tropical regions of Africa and Asia under scanty water conditions. Although pearl millet is grown in drought-prone semi-arid and arid areas of the world, it is also ideally adapted to irrigated farming. Pearl millet is an excellent forage crop because of its low hydrocyanic acid content. The green fodder which is rich in protein, calcium, phosphorous and other minerals contains oxalic acid within safe limits (Athwal and Gupta 1966 ; Gupta 1975). The major factors that restrict the production potential of pearl millet are low hybrid coverage, slow varietal spread and diseases (Gill 1991). These problems, however, can be overcome to some extent by diversification of male sterile lines, improvement of restorers and breeding heterogeneous and heterozygous single as well as multicross hybrids to combat the twin problems of low yield and diseases.

Pearl millet landraces

Large collection of pearl millet landraces from various states in India, is available at ICRISAT. The germplasm of pearl millet landraces shows a wide range of variation for several characters. Landraces from West Africa are in general very late maturing, tall, thick stalked with short spikes and bold grains. In Western India, the landraces are generally early-maturing, thin-stemmed and profusely branched, leading to asynchronous flowering, probably as an adaptive mechanism for low-rainfall conditions. Photoperiod sensitive types are present in hilly areas of Tamil Nadu and Andhra Pradesh. The lines collected from Tamil

Nadu contain more soluble sugars in their stalks during drought (Appa Rao *et al.* 1982).

Genetic diversity in pearl millet

Pearl millet cultivars have been generated from a narrow gene pool and current breeding programs do not make use of wild pearl millets. Genetic diversity studies in *Pennisetum* germplasm offer possibilities for their use in improving pearl millet varieties. In pearl millet, limited efforts have been made to study genetic diversity. The genetic differences have been studied by morphological and isozyme analysis (Tostain *et al.* 1987, Tostain and Marchais 1989 and Tostain 1992). Chloroplast DNA polymorphism has been found to be very less among several wild and cultivated millet samples (Clegg *et al.* 1984). Using ribosomal DNA sequences, greater diversity is observed in wild millets than in cultivated millets (Gepts and Clegg 1989). The mitochondrial DNA sequences are shown to be polymorphic in different cytoplasmic male sterile and fertile cytoplasms of pearl millet (Smith and Chowdhury 1989). Restriction Fragment Length Polymorphisms (RFLPs) have been shown to be highly polymorphic in pearl millet species (Liu *et al.* 1994).

In the present study, we show the usefulness of micro and minisatellites as well as RAPD markers to assess the genetic variation in Indian pearl millet cultivars and landraces for the first time.

MATERIALS AND METHODS

Plant material : Twenty two pearl millet cultivars and thirty six landraces (Table 1 and 2) were collected from IARI, New Delhi and ICRISAT, Hyderabad, India.

DNA analysis : Genomic DNA was extracted from young leaves of various genotypes as described by Sharp *et al.* (1988). DNA was digested with an excess of appropriate restriction enzyme (6-8 units/ug) under the conditions

recommended by the suppliers, separated on 1% or 1.4% agarose gels in 1xTPE buffer (0.09M Tris Phosphate, 0.002M EDTA, pH 8.0) and transferred onto nylon membranes. Alternatively, gels were dried on a vacuum gel dryer.

Table1 : Pearl millet cultivars used in the present study

Name	Characteristics
Tift23A	First line introduced in India from USA, downy mildew susceptible
5141A	Best general combiner, tillering type, medium size, compact earheads
5054A	Early, tillering type, narrow leaves
L-111-A	Late, long earheads, broad leaves
81A	Dwarf, tillering type
843A	Very early, dwarf
863A	Non tillering, purple glumes
841A	Derivative of MS-5141,medium size, compact earheads
3383A	Tillering type, dwarf
5122A	Dwarf, long earheads
5644A	Highly tillering, very thin stem,small earheads
189A	Tall, compact bold earheads
393A	Loose earhead
267A	Derivation of MS5141 through backcrossing with DM donors
ICMB88004	High tillering, yellowish green leaves
J-104	Best general combiner, drought tolerant, downy mildew susceptible
K-560-230	Tall, fast growing, broad leaves
D-23	DMR derivative of K-560-230
PPMI-301	Bold earheads
PPMI-493	Compact bold earheads
J-254	DMR derivative of J-104
ICMP-451	Tall, bold , semicompact bristled earheads

Table2 : Pearl millet landraces used in the present study

Sample No.	Genotype	State	Sample No.	Genotype	State
1.	IP6901	Uttar Pradesh	19	IP3126	Rajasthan
2	IP7251	"	20	IP3114	"
3	IP7093	"	21	IP3143	"
4	IP7346	"	22	IP3348	"
5	IP7133	"	23	IP4138	Maharashtra
6	IP4593	Madhya Pradesh	24	IP17876	"
7	IP4668	"	25	IP4195	"
8	IP13535	"	26	IP4147	"
9	IP4708	"	27	IP4197	"
10	IP3516	Tamil Nadu	28	IP15098	Karnataka
11	IP3522	"	29	IP15065	"
12	IP13601	"	30	IP15075	"
13	IP3605	"	31	IP15056	"
14	IP3652	"	32	IP11796	Andhra Pradesh
15	IP3762	Gujarat	33	IP8521	"
16	IP4021	"	34	IP16282	"
17	IP3807	"	35	IP3018	"
18	IP3738	"	36	IP8532	"

DNA probes and hybridization conditions : A human minisatellite sequence, pV47 (Longmire *et al.* 1990) and a 282bp fragment containing nine tandem repeats of 15bp core sequence from M13mp10RF(Vassart *et al.* 1987) were used in this study and were labeled using αP^{32} dNTP as described by Sambrook *et al.* (1989). The hybridizations were carried out for 16hrs in 40% formamide, 5xSSPE, 0.5%SDS, 5xDenhardt's and 0.1x BLOTTO at 42°C. The filters were washed in 1xSSPE, 0.5%SDS for 20 minutes at RT twice and hot wash was given at 55^{0}C for 15 minutes. Oligonucleotides were synthesized on gene assembler plus (Pharmacia), desalted on NAP-5 columns and purified on 20% denaturing polyacrylamide gel. Oligonucleotide probes such as *chi* sequence of *E.coli* (Ehtesham *et al.* 1992) ; repeats in the *Per* gene of Drosophila (Ben-Shlomo *et al.* 1993), the 3' hypervariable region of apolipoprotein B (Huang and Breslow 1987) and various di-, tri- and tetranucleotides were end labeled using γP^{32} ATP as described by Sambrook *et al* (1989). Hybridizations were performed at Tm-5^{0}C and were first washed with 5xSSPE, 0.1%SDS twice for 15 minutes at RT and then at the hybridization temperature for 2 minutes and were exposed to X-ray films at -70°C with intensifying screens.

PCR amplification : Operon series OPA, OPB and OPK (Operon Technologies Inc. Alameda, USA) were used and PCR was performed in volumes of $25\mu l$ consisting of $100\mu M$ each of dATP, dTTP, dCTP and dGTP, 5pmoles of primer, 20ng of genomic DNA, 1xreaction buffer and 0.5Units of Taq polymerase. Samples were amplified in a Perkin Elmer Cetus 48-well DNA thermal cycler programmed with a 3 minutes step at 94^{0}C for initial denaturation. This was followed by 45 cycles of 1 minute at 94^{0}C, 1 minute at 36^{0}C and 2 minutes at 72^{0}C. The final cycle was followed by a 5 minutes final extension step at 72^{0}C. Amplified products were separated on 1.4% agarose gels in 1xTAE buffer.

Data analysis : Each cultivar was scored for the presence or absence of reproducible bands and the data entered into a binary data matrix. Coefficients of similarity by simple matching were calculated and cluster analysis was performed using the UPGMA method. Dendrogram was produced using Taxan 4.0 program.

RESULTS AND DISCUSSION

Occurrence and abundance of hypervariable DNA sequences in pearl millet genome

Table 3 depicts the occurrence and polymorphism of di-, tri-, tetranucleotide and minisatellite repeats in the pearl millet genome. Among the tested micro and minisatellites, $(GATA)_4$ and pV47 generated most polymorphic patterns, both with cultivars and landraces (Table 3). Signals were obtained with all the probes except for $(AT)_{10}$, $(GC)_{10}$, $(ACG)_5$, $(ACC)_5$, 3'hypervariable region in Apolipoprotein, telomere and *Per* DNA sequences. Although other DNA markers produced many bands, number of polymorphic bands was either less or they were monomorphic or very faint. A few microsatellites such as $(GAA)_6$ and $(TG)_{10}$ produced very high number of bands, some of which were polymorphic (Table 3), however, heavy background smears were observed in both the cases inspite of stringent washing. Interestingly, microsatellites such as $(GATA)_4$, $(CAC)_5$ and $(GAA)_6$ produced high number of bands with all the three restriction enzymes *DraI, HaeIII and HinfI* (Table 3), indicating that most of the bands detected by hybridization were derived from separate loci in the genome, rather than from internal cuts within a cluster of repeats (Figures 1, 2 and 3). Earlier in chickpea, Sharma *et al.* (1995) have shown that $(GATA)_4$ hybridization bands were derived from different loci in the genome. Several initial investigations of plant microsatellites in barley, soybean, wheat, rice and tropical trees have demonstrated their informativeness and random distribution in plant species (Wang *et al.* 1994a).

Table 3 Occurrence and polymorphism of di-, tri-, tetranucleotides and minisatellite repeats in the pearl millet genome . (a) with cultivars (b), (c)and (d) with landraces

a] With cultivars

Probe	Total no.of bands			Total no. of polymorphic bands		
	*Dra*I	*Hinf*I	*Hae*III	*Dra*I	*Hinf*I	*Hae*III
(GATA)$_4$	21	19	18	20	19	18
(GGAT)$_4$	-	14	16	-	0	0
(GACA)$_4$	-	-	13	-	-	4
(CAC)$_5$	19	12	20	5	0	3
(GAA)$_6$	33	23	20	8	3	0
(TG)$_{10}$	30	-	-	8	-	-
pV47	21	27	-	6	15	1-
M13	-	18	-	-	12	-

b] Probes which generated polymorphic bands in landraces : (GATA)$_4$ and pV47

c]Probes which generated high no. of bands which were mostly monomorphic : (AG)$_{10}$, (AAG)$_5$,(AAC)$_5$, (ATG)$_5$, (ATC)$_5$, (AGA)$_5$ (AGT)$_5$, (AGG)$_5$, (AGC)$_5$, (ACA)$_5$, (ACT)$_5$, chi and M13 oligonucleotides

d] Probes which generated very faint bands or no signal:(AT)$_{10}$,(GC)$_{10}$,(ACG)$_5$, (ACC)$_5$, Apolipoprotein, Telomere and *per* DNA sequences

Diffuse background regions were observed previously in case of rice with a microsatellite $(TG)_{10}$, indicating the presence of large number of loci containing TG repeats (Ramakrishna et al. 1994). This was also reported earlier when DNA fingerprinting in plants was compared to that in humans (Rogstad et al. 1988). Background cross-hybridizing sequences indicate that either they are divergent from mini- or microsatellite repeats or fewer subrepeats are present. Compared to microsatellites, the bands obtained with pV47 were clear and polymorphic between 3kb to 6kb region and diffused below 3kb (figure 4) thus indicating that minisatellites are dispersed and clustered at certain loci in the genome.

DNA fingerprinting using $(GATA)_4$ microsatellite

Southern analysis using twenty two oligonucleotide probes and five minisatellite probes identified $(GATA)_4$ and pV47 as useful probes for the detection of multiple polymorphic fragments among pearl millet cultivars and landraces. Thirteen cultivar DNAs digested with DraI, HaeIII and HinfI and hybridized with $(GATA)_4$ probe exhibited unique DNA fragment profiles (Figures 1, 2 and 3). In all the three figures, 12 to 19 clear, resolvable bands were observed per lane. Figure 1 shows maximum number of bands per lane and the average number of polymorphic bands between pairs with standard deviation 16.4±5.16 with DraI enzyme . Figures 2 and 3 indicate distinct bands between 4Kb to 6Kb and more background smear with bands below 4Kb. The smear may be a result of the presence of a high number of different loci with short repeats. The cultivars 841B (lane7), 3383B (lane8) and 5122B (lane9) show less number of bands compared to other genotypes in Figures 1 and 2, whereas, with HinfI enzyme they show almost equal number of bands as the other cultivars (Fig. 3).

Figure 1 Hybridization patterns of *Dra*I digests of pearl millet DNAs detected by probe (GATA)$_4$. Lanes 1 to 12 are Tift23A(1), 5141A(2), 5054A(3), L-III-A(4), 81A(5), 863A(6), 841A(7), 3383A(8), 5122A(9), 5644A(10), 393A(11) and 267A(12). DNA marker is λ/*Hind*III digest.
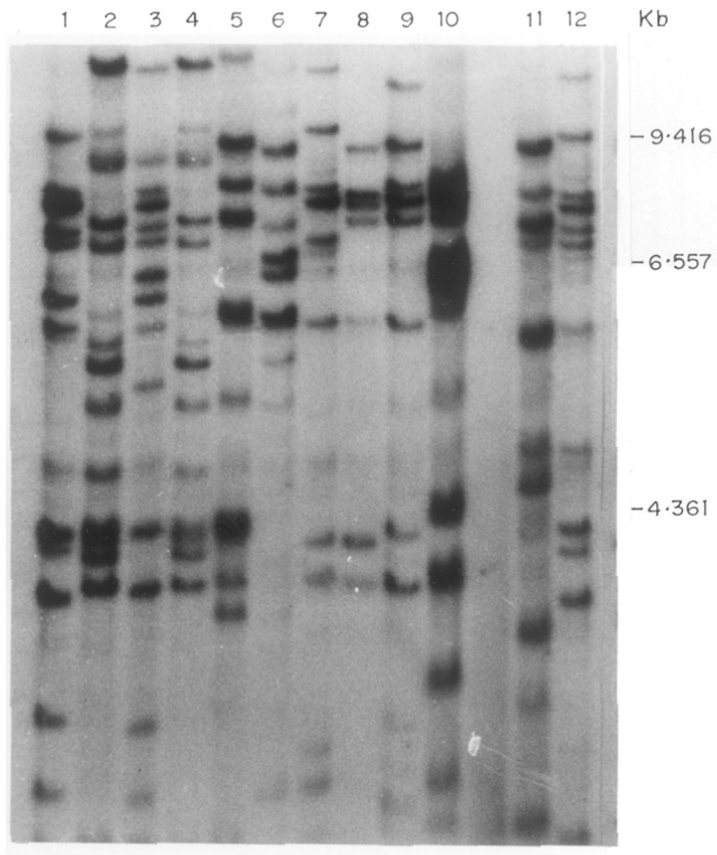
Fig.1

Figure 2 Hybridization patterns of *Hin*fl digests of pearl millet DNAs detected by probe (GATA)$_4$. Lanes 1 to 10 as in fig.1, 189A(11), 393A(12) and 267A(13). DNA marker is λ/*Hind*III digest.
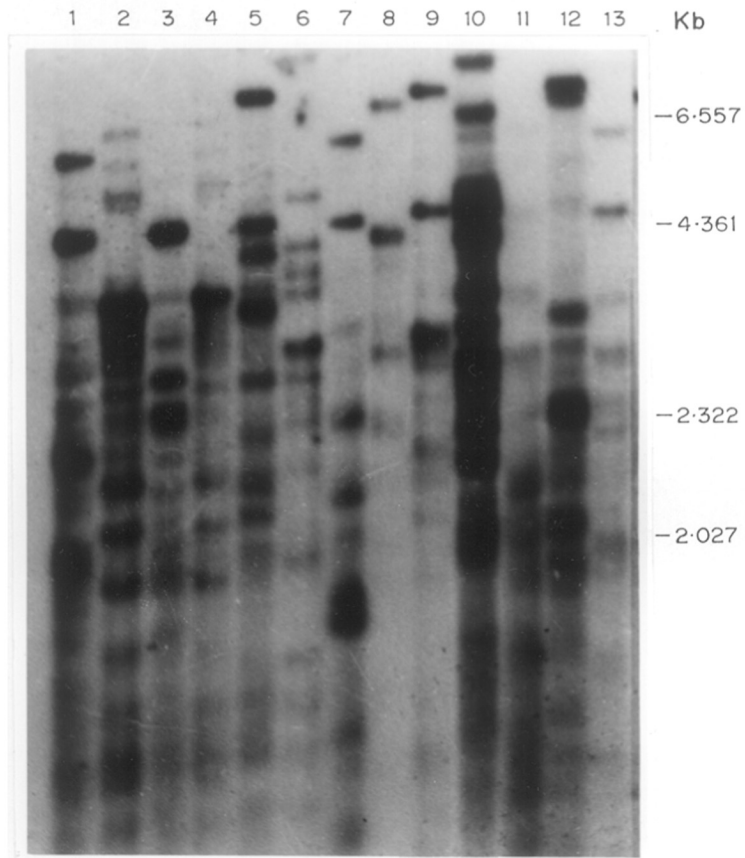
Fig. 2

Figure 3 Hybridization patterns of *Hae*III digests of pearl millet DNAs detected by probe (GATA)$_4$. Lanes 1 to 8 as in fig.1, 5644A(9), 189A(10), 393A(11) and 267A(12). DNA marker is λ/*Hind*III digest.
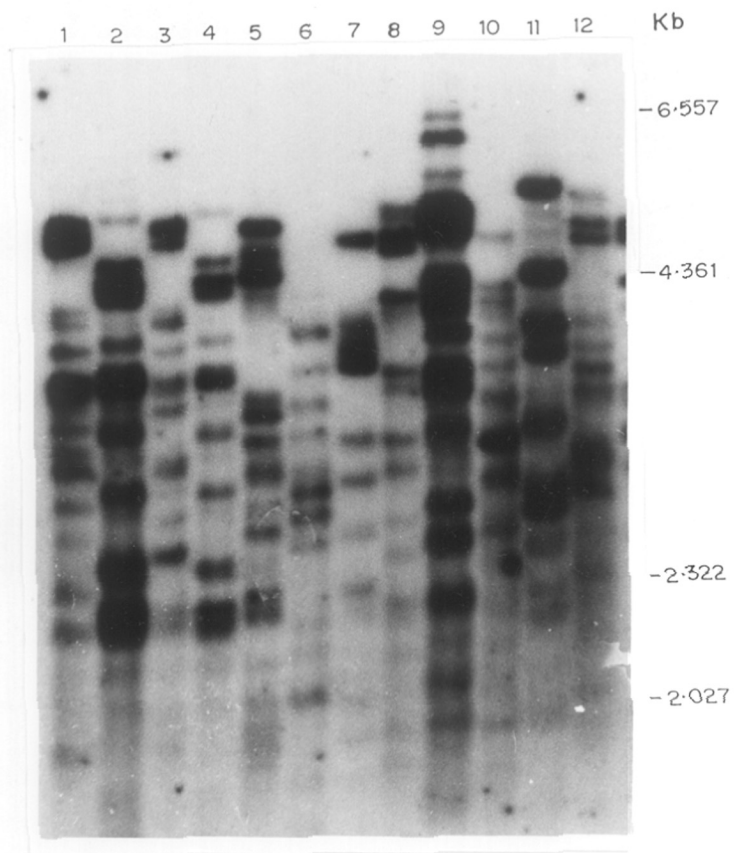
Fig. 3

Figure 4 Hybridization patterns of *Hinf*I (lanes 1 to 6) and *Sau*3AI (lanes 7 to 12) digests of pearl millet DNAs detected by probe pV47 Lanes 1 to 6 are 5054A(1), 81A(2), L III A(3), K-560-230(4), PPMI 301(5) and ICMP451(6). Lanes 7 to 12 are same as lanes 1 to 6. DNA marker is λ/*Hind*III digest.
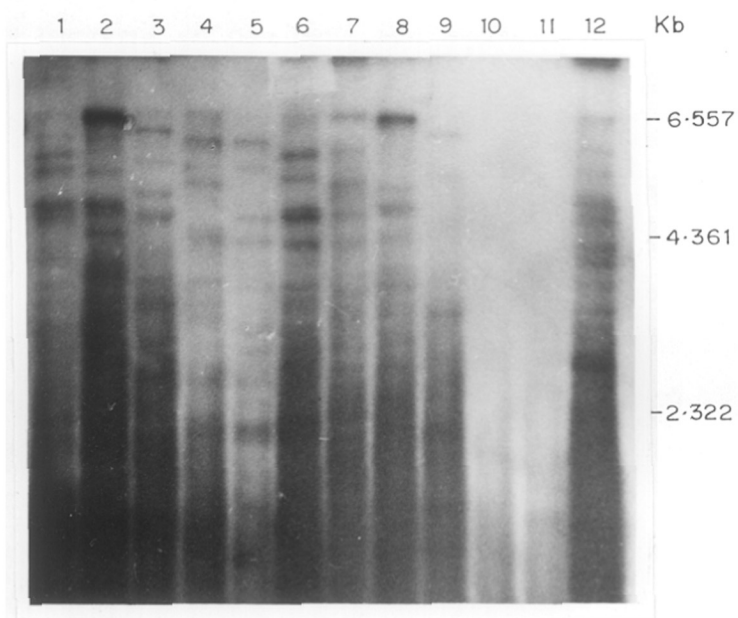
Fig. 4

Similarity index values for $(GATA)_4$ derived fingerprint patterns were calculated for all possible pairwise comparisons which ranged from 0.33 (Tift 23A compared with 5141A) to 0.65 (5644A compared with 5054A). The average similarity index values were lower for *Hinf*I (0.33) compared to *Dra*I (0.47) in case of cultivars. In landraces the similarity coefficient values ranged from 0.64 (IP6901[U.P] compared with IP7251[U.P]) to 0.90 (IP7093[U.P] compared with IP3762[Gujarat]). The average similarity index values were much higher for landraces compared to cultivars indicating more variability at $(GATA)_n$ containing loci in cultivars as compared to landraces of pearl millet. This may be due to the stable nature of landraces on one hand and recombinations created while developing cultivars in order to pyramid desired genes on the other hand. We have estimated the probability of identical fingerprints by chance to be 7.78×10^{-6}, 5.87×10^{-5} and 1.41×10^{-6} for cultivars with $(GATA)_4$ in combination with *Dra*I, *Hae*III and *Hinf*I, respectively (Table 4). Thus mean probability that two pearl millet cultivars have identical fingerprints with $(GATA)_4$ alone is 6.4×10^{-18}. However, the mean probability that two pearl millet landraces have identical fingerprints is very high and the landraces cannot be distinguished completely with $(GATA)_n$ probe alone, since the collection of pearl millet landraces at ICRISAT, Hyderabad alone is more than 40,000.

Thus our results indicate that $(GATA)_4$ has good potential as a fingerprinting probe in pearl millet cultivars. Similar results have been obtained in other crops such as rice (Ramakrishna *et al.* 1994), chickpea (Weising *et al.* 1992) and tomato (Vosman *et al.* 1992) where $(GATA)_n$ in combination with various restriction enzymes produces informative fingerprint patterns. Use of this microsatellite as a PCR primer can also be attempted, as tetranucleotide repeat primers have been shown to be most effective in amplifying polymorphic patterns (Gupta *et al.* 1994).

Table 4 Analysis of DNA fingerprints in cultivars[a] and in landraces[b]of
pearl millet

	DraI/GATA	HaeIII/GATA	HinfI/GATA	RAPDs
1.Average no.of bands	15.58 ± 3.5^{a}	11.23 ± 1.78^{a}	12.15 ± 3.1^{a}	$4.28\pm1.12/^{a}$
(n) ± SD	11.00 ± 1.7^{b}	--	5.61 ± 1.33^{b}	$3.4\pm0.43/^{b}$
2.No. of comparisons	66^{a}	78^{a}	78^{a}	209^{a}
	153^{b}	--	153^{b}	153^{b}
3.Average no.of polymorphic	16.4 ± 5.16^{a}	12.9 ± 3.35^{a}	15.9 ± 4.0^{a}	21.42 ± 5.62^{a}
bands between pairs ±SD	4.88 ± 2.9^{b}	--	4.72 ± 2.26^{b}	30.66 ± 3.94^{b}
4.Average similarity index	0.47 ± 0.14^{a}	0.42 ± 0.14^{a}	0.33 ± 0.15^{a}	0.628 ± 0.08^{a}
(X_D) ±SD	0.78 ± 0.12^{b}	--	0.53 ± 0.11^{b}	0.66 ± 0.068^{b}
5.Probability of identical	$7.78\times10^{-6\,a}$	$5.87\times10^{-5\,a}$	$1.41\times10^{-6\,a}$	$4.7\times10^{-5\,a}$
match by chance $(X_D)^{n}$	$6.5\times10^{-2\,b}$	--	$2.8\times10^{-2\,b}$	$2.9\times10^{-6\,b}$

*The values indicated for RAPD were obtained with 5 and 9 primers in cultivars
and landraces, respectively.

DNA fingerprinting using RAPD markers

RAPD analysis was performed with 20 primers on 21 cultivars and 60
primers on 36 landraces. The primers giving inconsistent polymorphic patterns
in various runs were eliminated from our analysis. All the RAPD primers used
resulted in the amplification of 1 to 10 bands with an average of 8 bands per
primer in cultivars and 5 in case of landraces. OPA10, OPA12, OPA16, OPA19,
OPA20 and OPB12 generated polymorphic banding patterns in cultivars . In
landraces four different region specific genotypes were taken for initial screening
to identify informative primers. OPA4, OPA12, OPA14, OPA16, OPB5, OPB8,
OPB12 and OPB20 were found to be polymorphic. The polymorphism detected
in cultivars and in landraces is represented in Figures 5, 6, 7 and 8 using
primers OPA10, OPA12, OPA16, OPA20, OPB5, and OPB20. In general, the

extent of polymorphism generated by each primer was different in cultivars and in landraces (Table 5). This might be because of out-crossing in the present cultivars and more inbreeding in landraces. Based on the polymorphic patterns obtained, similarity index values were calculated, which ranged from 0.54 to 0.86 for cultivars and 0.64 to 0.85 for landraces. The average similarity index values were almost similar for both (Table 4) and the probability of identical match by chance per primer was 1.3×10^{-1} for cultivars and 2.4×10^{-1} for landraces. Eventhough, the values are higher compared to $(GATA)_n$, RAPDs can identify greater number of genotypes by including more primers.

Table 5 Amplification patterns of pearl millet DNA with Operon primers

Primer	Total no. of bands		Total no ofpolymorphic bands	
	cultivars	landraces	cultivars	landraces
1.OPA4	--	8	--	7
2.OPA10	9	--	3	--
3.OPA12	10	9	10	2
4.OPA14	--	6	--	2
5.OPA16	12	12	7	8
6.OPA19	10	--	2	--
7.OPA20	15	--	15	--
8.OPB5	--	9	--	9
9.OPB12	11	10	11	4
10.OPB20	--	10	--	10

Figure 5A and 5B RAPD fingerprinting of pearl millet genotypes with primer OPA 10 and OPA 12. Lane M is ϕX174/*Hae*III digest marker. Lanes 1 to 23 are 23A(1), 5141A(2), 5054A(3), L-III-A(4), 81A(5), 843A(6), 863A(7), 841A(8), 3383A(9), 5122A(10), 5644A(11), 189A(12), 490A(13), 393A(14),267A(15), ICMB88004(16), J104(17), K-560-230918), D23(19), PPMI301(20), PPMI493(21), J254(22) and ICMP451(23).
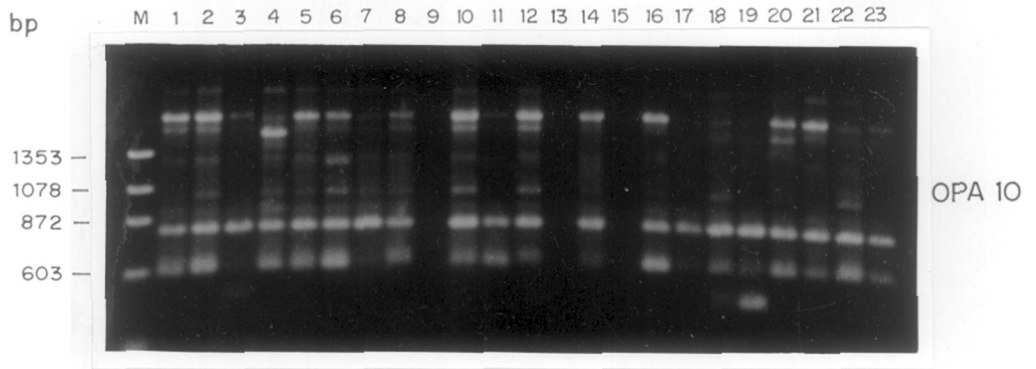
Fig. 5A
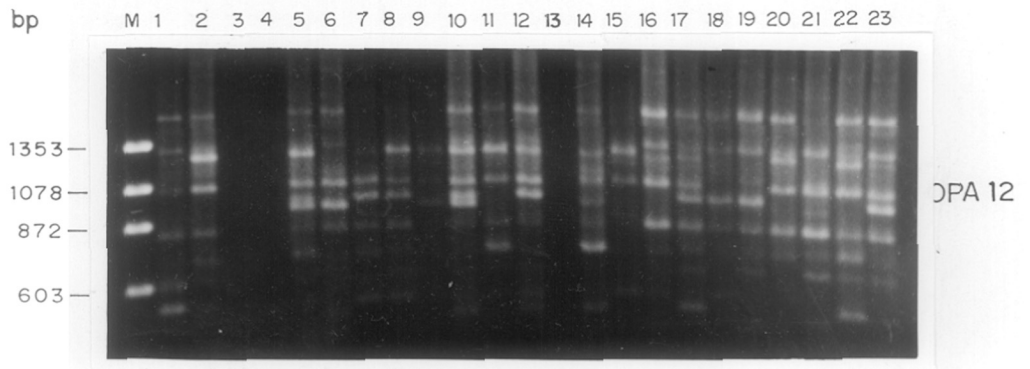
Fig. 5B

Figure 6A and 6B RAPD fingerprinting of pearl millet genotypes with primer OPA 16 and OPA20. Lanes 1 to 23 are as shown in Figure 5.

Fig. 6A

Fig. 6B

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Figure 7A and 7B RAPD fingerprinting of pearl millet landraces with primer OPB. 5. Lanes 1 to 36 are as shown in Table 2. Molecular weight marker is ϕX174/*Hae*III digest.

Fig.7A
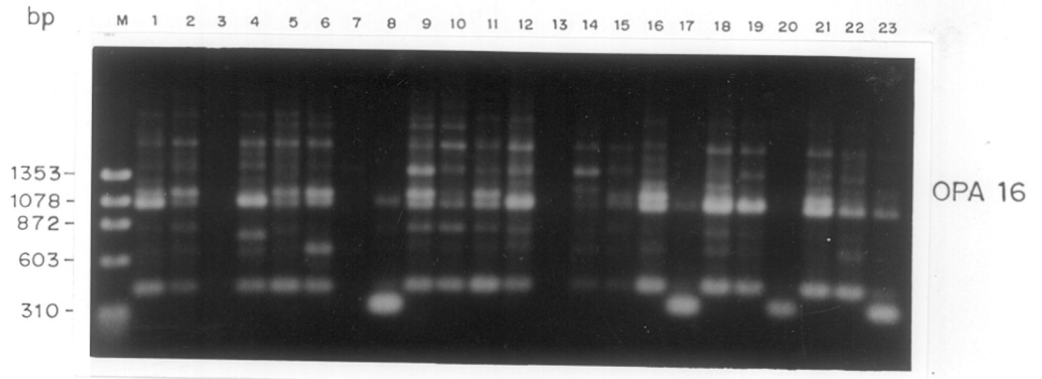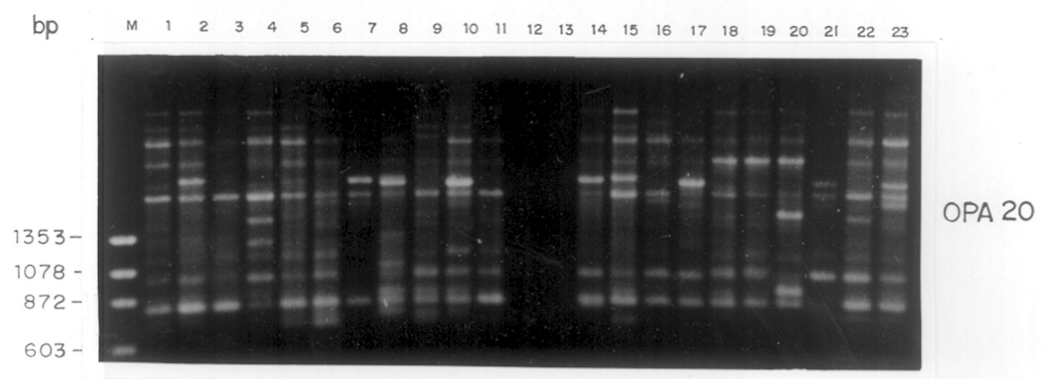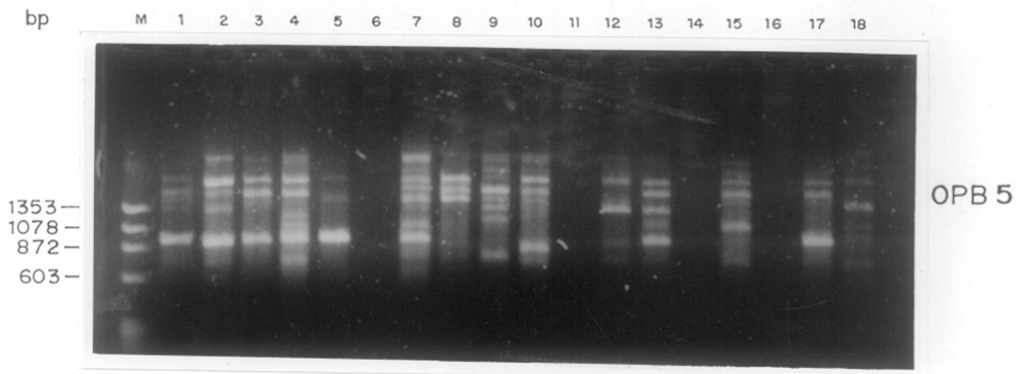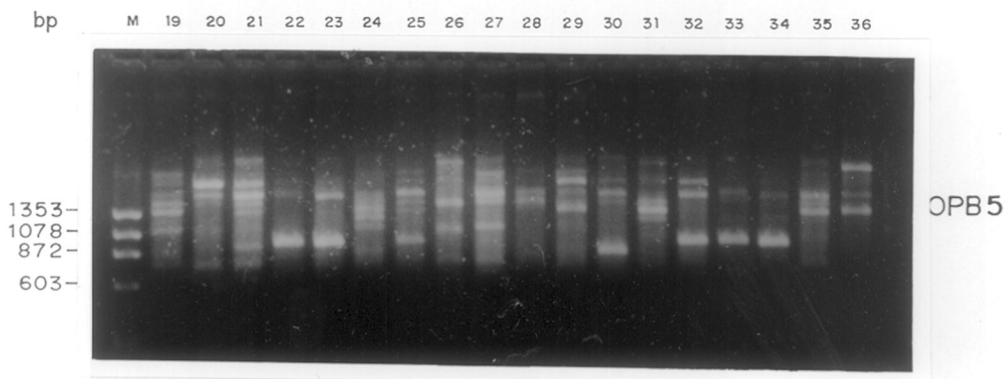

Fig.7B

Figure 8A and 8B RAPD fingerprinting of pearl millet landraces with primer OPB. 20. Lanes 1 to 36 are as shown in Table 2. Molecular weight marker is ϕX174/*Hae*III digest.

Fig. 8A

Fig. 8B

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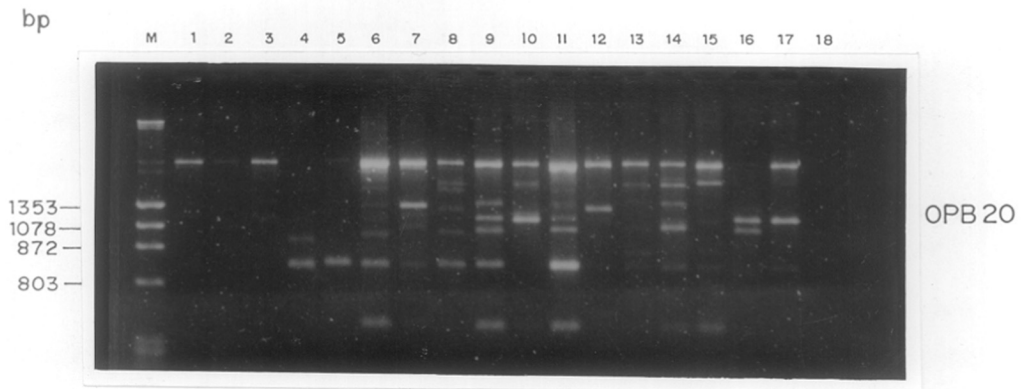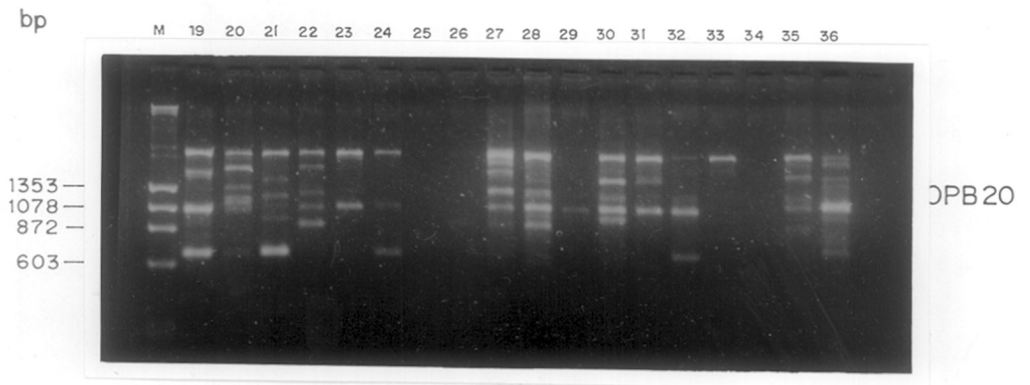
Problems with reproducibility of RAPD banding patterns have been reported by Penner *et al* (1993). In our laboratory they were solved by optimizing the conditions, running replications for screening primers and discarding primers which generated inconsistent polymorphic banding patterns. In addition only strong, reproducible bands were used in the analysis while variable, faint bands and those above 2kb which were not repeatable were excluded. The RAPD analysis revealed a high degree of genetic diversity among the cultivars as well as landraces used in the study.

Genetic relationships

The grouping of 15 cultivars (LxT mating design) based on D^2 statistics is as shown in Table 6. Although 841A, 267A and 5141A were grouped under the same cluster, J104 and its derivative J254 represented two different clusters on the basis of D^2 statistics. Clusters generated using $(GATA)_n$ and RAPD data were also not completely in agreement with the available pedigree data. All the three genotypes 841A, 5141A and 267A were grouped in different clusters based on $(GATA)_4$ as well as RAPD data (Fig 9a and 9b). Genotypes J254 and J104 were present in two different clusters while K-560-230 and D-23 formed one cluster based on RAPD data (Fig 9b). The two cultivars 5141A and L-III-A having 97% similarity with $(GATA)_n$ hybridization (Fig 9a) showed no striking similarity with RAPDs (Fig 9b). In general, the groups established by D^2 analysis and clusters developed by $(GATA)n$ and RAPD analysis did not show much correlation. Interestingly, the landraces representing eight states of India also could not be grouped based on their geographical distribution using DNA marker data (Fig 10).

In the present study pedigree data were not available for all cultivars. Tift23A, the first line inroduced in India, shares common genetic background with most of the present CMS lines. MS-5141 was derived from Tift23 by backcrossing program (Pokhriyal *et al*. 1976). MS-841 was derived from a cross

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between MS-5141 and unknown downy mildew resistant source (Singh *et al.* 1990). MS-267 was developed from 5141 through backcross breeding program utilizing 700651 downy mildew resistant donor from West Africa. MS-81 was

Table 6 Grouping of 15 parents (LxTmating design) into clusters based on D^2 statistics

Cluster	No.of inbreds	Name of the inbreds
I	6	267A,5141A,81A,393A,Tift23A,841A
II	2	J104,843A
III	4	PPMI493,189A,ICMP451,PPMI-301
IV	1	J-254
V	1	863A
VI	1	D-23

a simple mutation of the Tift23A (Kumar *et al.* 1984). D-23 was a reselection from K-560-230 and J-254 was an end product of backcross program from J-104 utilizing very diverse downy mildew resistant source from West Africa. The groups MS-5141A, 841A, 267A and 81A; K-560-230 and D-23; J-104 and J-254 share a common genetic background but in view of their pedigrees it cannot be assumed that they are genetically similar. In fact they differ in their combining ability (unpublished data). Out-crossing might be a reason for large number of morphological changes in these cultivars (Rai and Hanna 1990).

Figure 9a : Genetic relationships among pearl millet cultivars using
(GATA)$_4$ oligonucleotide as a probe with *Dra*I, *Hinf*I and
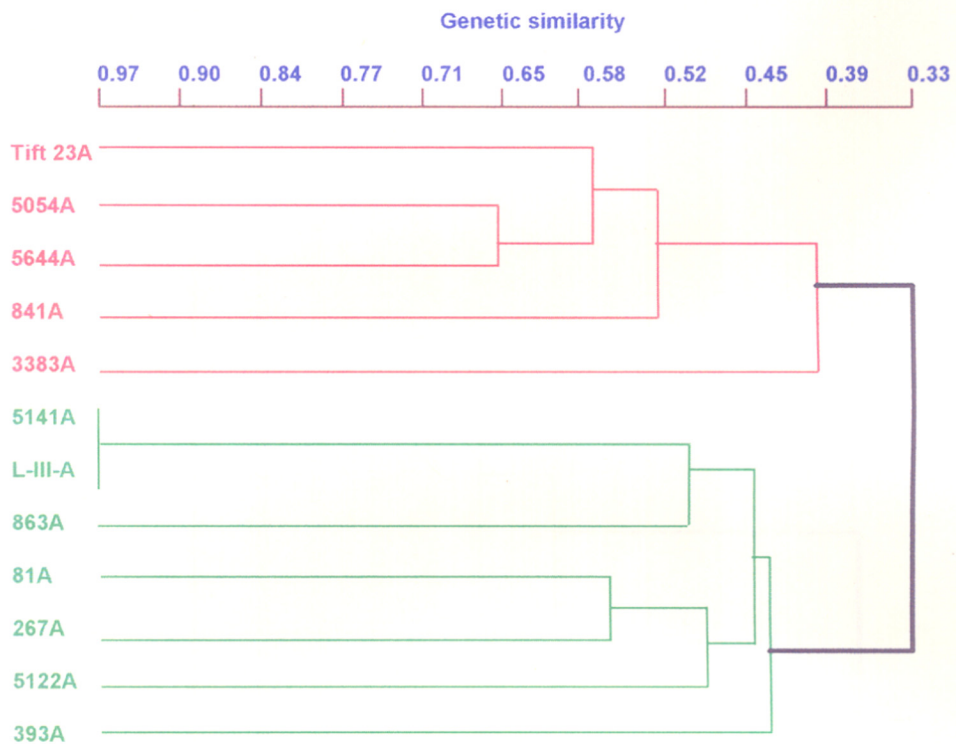*Hae*III restriction enzymes

Figure 9b : Genetic relationships among pearl millet cultivars using RAPD primers
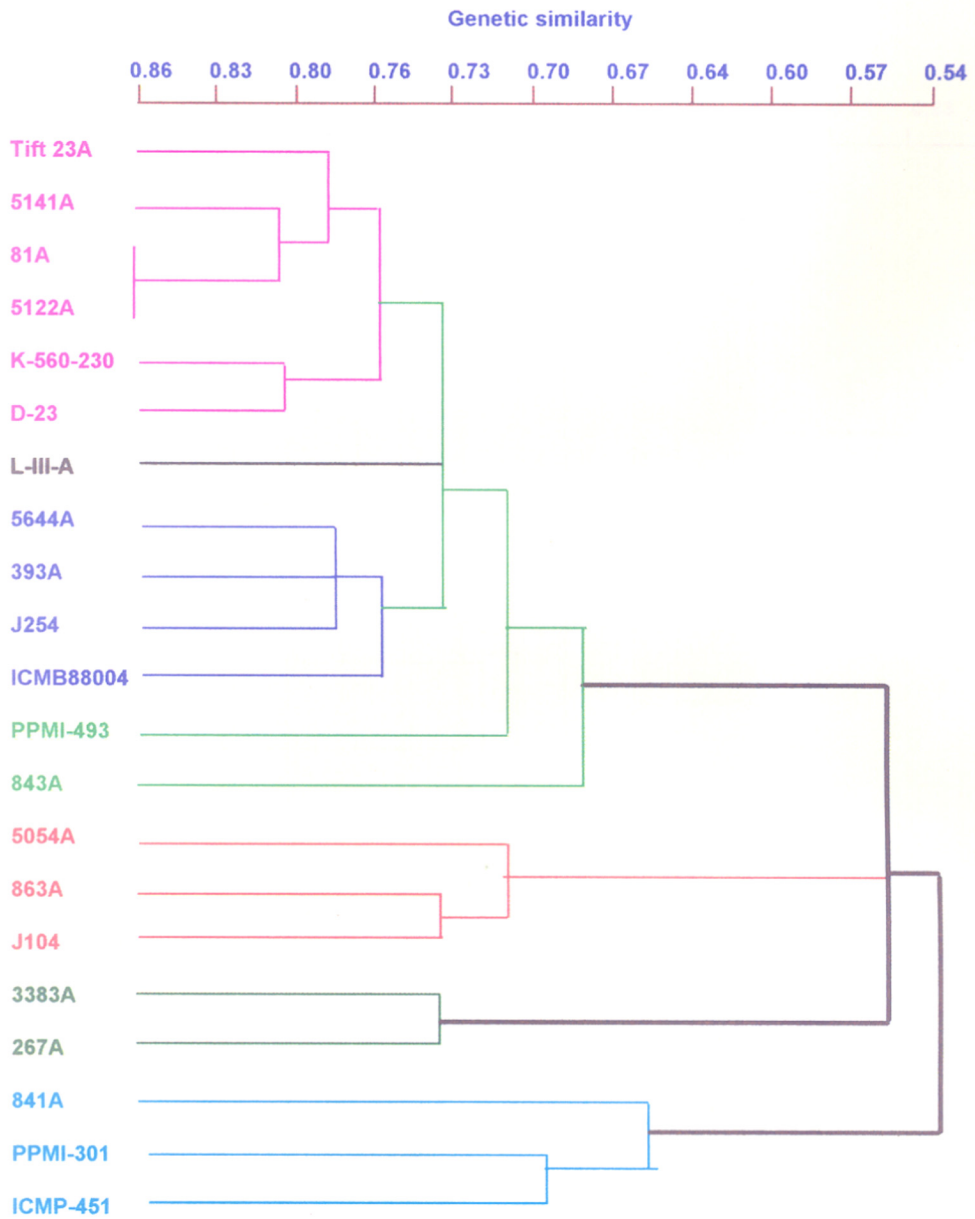
Figure 10 : Genetic relationships among pearl millet landraces using RAPD primers. Numbers 1 to 18 represent as in Table 2
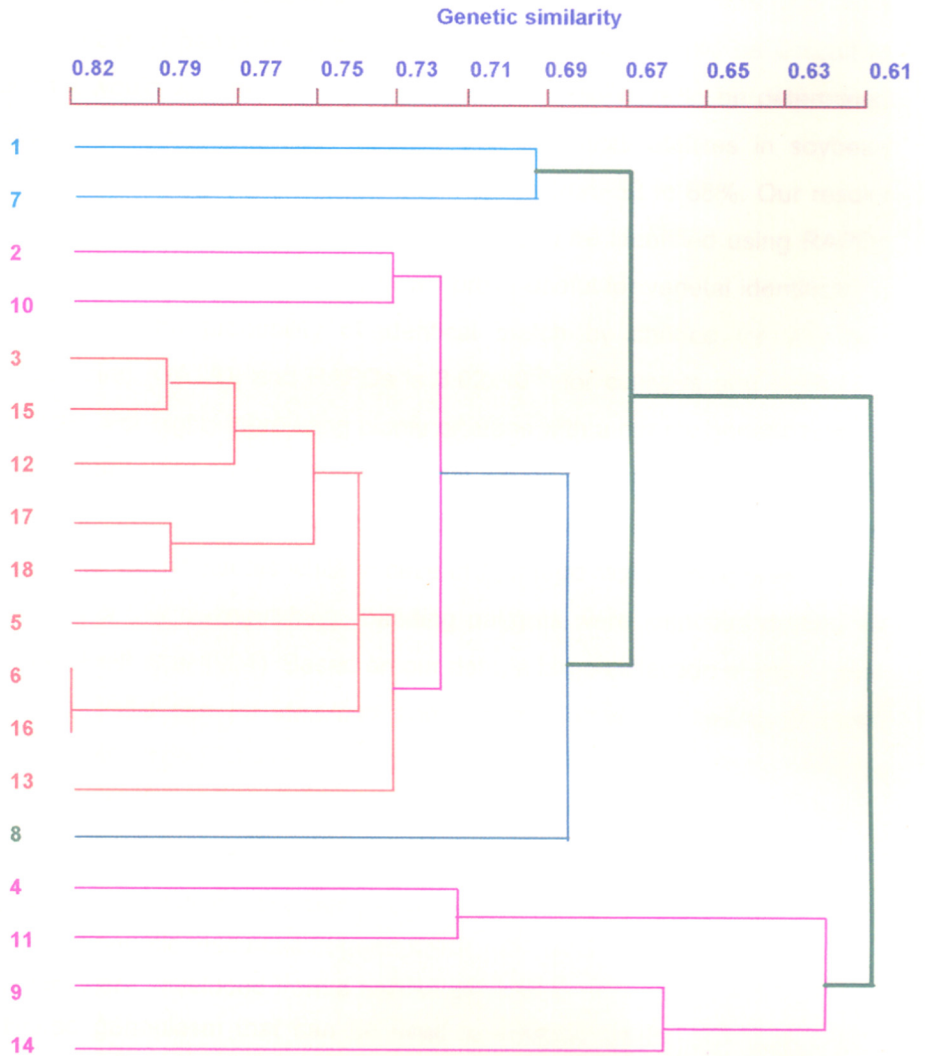
Importance of (GATA)$_4$ and RAPD data in pearl millet

Several studies have clearly demonstrated the utility of RAPDs and microsatellites for identifying plant varieties (Morrel *et al* 1995; Akkaya *et al* 1992; Rongwen 1994). Earlier results indicated that the exact number of primers and the number of bands required to distinguish varieties would be difficult to predict as it would vary from group to group and would have to be determined empirically. Akkaya *et al* (1992) reported that the microsatellites in soybean could detect heterozygosity per locus which varied from 52 to 88%. Our results have shown that all the cultivars and landraces can be identified using RAPDs and GATA hybridization patterns which will prove useful for varietal identification in pearl millet. The probability of identical match by chance for any two genotypes using (GATA)$_n$ and RAPDs is 3.02×10^{-24} for cultivars and 0.52×10^{-12} for landraces. For highly inbreeding plants or crops with a narrow genetic base, it is more difficult to find probes or primers that will discriminate the genotypes. In case of the out crossed species *Plantago lanceolata*, M13 probe could discriminate all 32 individuals while in case of *Plantago major* which is an inbred species, only a few distinguishing banding patterns were identified among 40 individuals (Wolff *et al* 1994). Based on our data, a large collection of pearl millet landraces in India can be identified and exploited in the breeding program including identification of duplicates in germplasm collections. The loci containing many types of SSRs as shown by our studies can be effectively used by converting them into sequence tagged microsatellite sites (STMSs).

Success of crop improvement program largely rests on the availability and knowledge of genetic resources in germplasm collections. In case of pearl millet, the cultivars were developed from a narrow gene pool. Wild *Pennisetum* species offer diverse germplasm that can be used to improve pearl millet and useful characteristics for this purpose are disease and insect resistance , genes for fertility restoration of the A1 cytoplasm, cytoplasmic diversity, QTLs for yield, apomixis, maturity and many inflorescence and plant morphological

characteristics. At present not much effort has been made to look into primary, secondary and tertiary gene pools in *Pennisetum* using DNA markers. (GATA)$_4$ and RAPDs could be of great benefit in exploiting the wild germplasm during introgression breeding programs and identification of varieties having good combining abilities without the evaluation of F$_1$ hybrids in the field to predict heterotic combinations.

CHAPTER III

SECTION - B

HYBRID PERFORMANCE AND GENETIC

DISTANCE AS REVEALED BY (GATA)$_4$

MICROSATELLITE AND RAPD

MARKERS IN PEARL MILLET

INTRODUCTION

Pearl millet is a cross-pollinated crop and its genetic improvement has been carried out by developing hybrids through conventional breeding procedures. The average yield of pearl millet in India fluctuates around 500kg/ha which is extremely low compared with other kharif crops as well as pearl millet production in other places (Gill 1991). The primary reason of such a low yield is the cultivation of pearl millet on too poor or too dry soils where other cereals would fail to produce a crop. The improvement in yielding ability envisages the assembling of the best combination of yield genes into a pearl millet variety or hybrid along with the improvement of performance under natural conditions. Pearl millet is highly heterozygous because of the cross-pollinating system of the crop. Recurrent selection is used to pool genes for a particular quantitative characteristic in an open-pollinated population, without a marked loss of genetic variability (Govil *et al* 1982).

The exploitation of cytoplasmic male-sterility in pearl millet hybrid development started in 1962 with the availability of Tift23A1 and indicated a great scope for the genetic upgradation of this crop. Heterosis which results from gene dispersion, directional dominance and non-allelic interactions is computed as deviation of the F_1 from the better parent which is sometimes referred to as heterobeltiosis. Heterosis over mid-parent corresponds to the dominance deviations on the scale of Mather (Mather and Jinks, 1971). It can be exploited commercially, for example, in pearl millet, single crosses have been used to exploit heterosis. Positive correlations between genetic distance of the parents with heterosis in hybrids were reported by Paterniani and Lonquist (1963), Moll *et al* (1965), Grant and Beversdorf (1985) and Lefort-Buson *et al* (1987). In another study, Prasad and Singh (1986) reported that heterosis was not linearly related to genetic divergence in a study of ten maize varieties.

Molecular markers can be effectively used to assess the genetic diversity precisely. RFLPs have been used in corn to study the relationship between genetic diversity and heterosis (Lee *et al* 1989; Melchinger *et al*

1990b; Smith *et al* 1990). In recent years, PCR-based markers (RAPDs) have been proved to be useful in the analysis of genetic diversity and its relationship to hybrid performance and heterosis in rice (Xiao *et al* 1996b). Apart from RFLP and RAPD, DNA fingerprinting with mini- and microsatellites can also be used to study the genetic diversity of parents to be used in breeding (Ramakrishna *et al* 1994 and 1995, Meng *et al* 1996). So far various contrasting results have been reported in relation to prediction of heterosis and in general the correlation values have been shown to be dependent on the type of germplasm used (Lee *et al* 1989; Godshalk *et al* 1990; Zhang *et al* 1994; 1995; 1996; Saghai Maroof *et al* 1997). Furthermore an increased correlation between marker heterozygosity and closely related germplasm has been reported by Zhang *et al* 1996.

In pearl millet so far combining ability studies were mostly carried out for morphological and biochemical characters and yield parameters. It was observed that the characters, tiller number, 500 grain weight, and plant height had high heritability and high genetic advance, which revealed effect of additive gene action. Highly significant and positive correlation for tiller number followed by 500 grain weight with yield, indicated major role of these characters in the contribution of yield (Kunjir *et al* 1986). So far there is no account of molecular markers for variability and correlation studies in pearl millet. In our earlier work (Chowdari *et al* 1998), we have shown the potential of (GATA)$_4$ microsatellite and RAPDs in the genetic diversity evaluation of pearl millet varieties and landraces. In the present study, we evaluated the pearl millet CMS and restorer lines for their hybrid performance and heterosis and their relation to genetic distance for prediction of heterotic combinations.

MATERIALS AND METHODS

Plant material and field evaluation : Seven cytoplasmic male sterile lines (81A, 863A, Tift23A, 5141A, 841A, 393A and 267A) and five restorers (D23, ICMP451, PPMI301, PPMI493 and J254) of pearl millet were used in the present study. The crossing work was carried out in off-season nursery at

Coimbatore, India during the summer of 1995. Bagging and pollination were carried out in the morning from 8 a.m. to12 noon. Thirty five F1 hybrids were developed and the experiments were laid out in a completely randomized block design during the kharif of 1995 at IARI, New Delhi, India. The crosses and parents were restricted to separate but consecutive blocks within each replication and randomization was done separately for crosses and parents. Agronomic practices were carried out in accordance with the recommendations to this area and season. A spacing of 75cm between the rows and 10cm between the plants was given. The traits evaluated were days to 50% flowering, plant height, productive tillers, ear length, ear width, 1000 grain weight and grain yield per plot.

DNA marker analysis : Twelve lines of pearl millet were surveyed for DNA polymorphism with 20 Operon primers (OPA1 to OPA20) from Operon Technologies and with $(GATA)_4$ microsatellite probe. The DNA extraction procedure followed was as described by Sharp *et al* (1988). DNA was digested with restriction enzymes *Dra*l, *Hinf*l and *Hae*lll (6-8 units/ug), separated on 1% or 1.4% agarose gels in 1xTPE buffer (0.09M Tris phosphate, 0.002M EDTA, pH 8.0) and gels were dried on a vacuum gel dryer. $(GATA)_4$ hybridization procedure in combination with *Dra* I, *Hinf* I and *Hae* IIIwas as described by Ramakrishna *et al* (1995). Hybridizations were performed at Tm-5°C and the hybridized gels were first washed with 5xSSPE, 0.1%SDS twice for 15 minutes at RT and then at the hybridization temperature for 2 minutes and were exposed to X-ray films at -70°C with intensifying screens.

RAPD - PCR analysis : PCR reactions were performed using Operon primers in volumes of 25 µl consisting of 100µM each of dATP, dTTP, dCTP and dGTP, 5pmoles of primer, 20ng of genomic DNA, 1xreaction buffer and 0.5U of Taq DNA polymerase (Biogenie, India). Samples were amplified in a Perkin Elmer Cetus 48-well DNA thermal cycler programmed with a 3 minutes step

at 94°C for initial denaturation. This was followed by 45 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C. The final cycle was followed by a 5 minutes final extension step at 72°C. Amplified products were separated on a 1.4% agarose gels in 1xTAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.0).

Field data analysis : The twelve lines in the experiment were assessed for all the seven characters by giving a final score for each line across the seven characters. To assess the crosses, the mean performance of the check Pusa 23 was taken as a standard. For each character, the overall mean was taken as a standard and those lines expressing values greater than the mean + standard error were assigned a high status (H) and others received the low status(L). Similarly for days to 50% flowering and plant height, the lines expressing values below the mean - standard error were considered desirable (D) and others as undesirable (UD). For the status of H or D, a score of +1 was given while L or UD received a zero score. For each line, a total score was obtained by adding the score across the characters. For combining ability analysis, only crosses were considered and analyzed based on the line x tester analysis. Heterosis for each cross was computed as a deviation from the mid-parent value.

Rank correlations were calculated between F1 performance, heterosis over midparent and better parent and the genetic distance.

RESULTS
Per se performance of parents and hybrids

The seven female lines and five testers in the experiment were assessed for all seven characters by giving a final score for each line across the seven characters as mentioned in Materials and Methods. The line 863A obtained the first rank with a score of five. The lines 81A and 393A stood second with a score of four each and the third place was occupied by 5141A with a score of three. 841A, with a score of 2 got fourth place whereas Tift23A

and 267A were placed in fifth position with a score of one. The tester PPMI301 ranked second with a score of four, however, it was undesirable both for days to 50% flowering and plant height. The three testers D23, ICMP451 and PPMI493 obtained third rank each with a score of three. The tester J254 was in fourth position with a score of two (Table 1).

Based on total score, 35 crosses between all CMS and tester lines were divided into five groups as described in Materials and Methods. Group I had maximum score(4) and group V had the minimum score(0). Only two crosses 841AxPPMI301 and 393AxJ254 occupied the group I and were desirable both for days to 50% flowering and for plant height. 81AxD23 cross got score of three out of maximum possible five for yield components. In group II, 6 crosses were grouped where as in group III, 16 crosses were placed. Nine crosses were placed in group IV (Table 2).

Heterosis over midparent and better parent is represented for all 35 crosses in Table 3. For days to 50% flowering, eight and two crosses recorded significant heterosis over midparent and better parent, respectively, in the desired direction (for earliness). The cross 393AxPPMI493 showed the maximum heterosis for earliness as -10.77% over midparent and -9.38% over better parent. For plant height, none of the crosses showed significant heterosis over midparent and better parent in negative direction (dwarfness). For productive tillers, 81AxD23 cross showed significant heterosis in desired direction with the maximum value of 125% over mid as well as better parent. For ear length and ear width, the crosses Tift23AxD23 and 393AxJ254 , respectively, showed significant heterosis over midparent and better parent compared with other crosses. For 1000 grain weight and grain yield, significant heterosis was found but none of the crosses performed significantly better than Pusa 23 (check) for 1000grain weight.

DNA polymorphism and cluster analysis

In the previous section, we have shown (GATA)$_4$ and RAPDs as useful DNA fingerprinting markers for the analysis of pearl millet genome (Chowdari

et al 1998). In the present study, twelve genotypes were analyzed with (GATA)$_4$ microsatellite using *Dra*I, HinfI and *Hae*III restriction enzymes and 20 Operon primers (OPA series). The twelve genotypes analyzed using microsatellite probe exhibited unique DNA fingerprint profiles and generated 37, 28 and 36 polymorphic loci with DraI , *Hinf*I and *Hae*III,respectively. Out of 20 primers tested, fourteen (OPA2,3,5,8,10 to 17, 19 and 20) were used for analysis and generated 59 polymorphic loci. OPA 20 was shown to be highly polymorphic and generated 14 polymorphic bands. Using (GATA)$_4$ and 14 operon primers, a total of 160 polymorphic loci were generated. Band profiles for each parent were designated as 1 for presence or 0 for absence of that band. Genetic similarities (GS) were calculated for all pairwise combinations of parents using methods described by Nei and Li (1979). Based on the polymorphism data, the similarity index values were calculated which ranged

Table 1 Score performance and overall status of parents based on *per se* performance (LxT analysis)

Parents	Score			
	Desirability	Yield components	Total	Rank
Lines				
81A	1	3	4	II
863A	1	4	5	I
Tift23A	1	0	1	V
5141A	0	3	3	III
841A	1	1	2	IV
393A	1	3	4	II
267A	0	1	1	V
Testers				
D23	1	2	3	III
ICMP451	0	3	3	III
PPMI301	0	4	4	II
PPMI493	0	3	3	III
J254	2	0	2	IV

Table 2 Score performance and over all status of hybrids based on *per se* performance in comparison with Pusa 23 (841A x D23)

Hybrids	Score			
	Desirability	Yield components	Total	Rank
81AxD23	0	3	3	II
81AxICMP451	0	1	1	IV
81AxPPMI301	0	2	2	III
81AxPPMI493	0	1	1	IV
81AxJ254	0	1	1	IV
863AxD23	1	1	2	III
863AxICMP451	1	1	2	III
863AxPPMI301	0	2	2	III
863AxPPMI493	0	2	2	III
863AxJ254	1	1	2	III
Tift23AxD23	1	1	2	III
Tift23AxICMP451	1	0	1	IV
Tift23AxPPMI301	1	2	3	II
Tift23AxPPMI493	1	2	3	II
Tift23AxJ254	2	0	2	III
5141AxD23	0	2	2	III
5141AxICMP451	0	1	1	IV
5141AxPPMI301	1	0	1	IV
5141AxPPMI493	0	2	2	III
5141AxJ254	0	0	0	V
841AxD23	check	check	check	check
841AxICMP451	0	1	1	IV
841AxPPMI301	2	2	4	I
841AxPPMI493	1	1	2	III
841AxJ254	2	0	2	III
393AxD23	1	1	2	III
393AxICMP451	2	1	3	II
393AxPPMI301	0	2	2	III
393AxPPMI493	1	2	3	II
393AxJ254	2	2	4	I
267AxD23	0	1	1	IV
267AxICMP451	1	1	2	III
267AxPPMI301	2	1	3	II
267AxPPMI493	0	2	2	III
267AxJ254	1	0	1	IV

Table 3 Heterosis over mid parent and better parent (L x T mating design)

		Days to 50% flowering		Plant height		Productive tillers		Ear length		Ear width		1000 grain weight		Grain yield per plot	
		MP	BP	MP	BP	MP	BP	MP	BP	MP	BP	MP	BP	MP	BP
1.	81AxD23	11.48**	20.57**	55.84**	22.95**	125.00**	125.00**	40.02**	24.88**	26.26**	5.72	13.89	0.35	157.25**	95.14**
2.	81AxICMP451	6.71**	12.08**	54.76**	33.33**	84.62*	71.43**	10.11*	7.27	6.10	-11.26**	5.49	-2.35	113.12**	66.46*
3.	81AxPPMI301	5.99**	9.80**	34.44**	10.50	25.00**	25.00	16.44**	13.89*	25.60**	4.11	26.07*	18.00	135.82**	56.20**
4.	81AxPPMI493	3.34*	3.66*	41.04**	19.23**	36.00**	30.77	21.02**	12.63*	7.79	-7.78	12.56	10.09	85.60*	34.62
5.	81AxJ254	13.71**	25.93**	57.65**	45.78**	85.10**	66.67**	26.44**	14.02*	9.23	-2.46	20.33	0.46	182.69**	150.21**
6.	863AxD23	4.86**	7.09**	25.11**	15.57**	-13.51	-36.00*	-0.32	-11.93**	13.47**	3.88	12.52	5.17	82.09**	71.43**
7	863AxICMP451	3.38*	4.08*	20.86**	24.51**	-23.00	7.14	3.45	1.85	-3.48	-11.51**	14.87	2.13	53.09*	50.00
8.	863AxPPMI301	7.33**	9.52**	19.40**	16.16**	2.70	-24.00	-0.40	-3.59	17.43**	8.76	25.73**	10.64	82.89**	42.27**
9.	863AxPPMI493	4.49*	10.88**	26.15**	26.09**	-21.00	-40.00**	-10.36*	-15.74**	8.61	-2.88	6.64	-9.73	125.93**	96.88**
10.	863AxJ254	9.93**	14.81	30.90**	17.99**	-25.00	0.00	7.09	-4.35	16.87**	0.13	25.05	-9.73	112.50**	87.06**
11.	Tift23AxD23	1.38	4.62*	11.70*	-1.80	38.46	28.57	46.80**	42.12**	21.47**	1.81	51.60**	7.34	115.66**	57.43*
12.	Tift23AxICMP451	4.70**	4.70**	21.58**	18.46**	21.43	28.43	19.93**	7.57	0.45	-15.92**	40.27**	2.73	145.55**	84.16**
13.	Tift23AxPPMI301	-0.66	0.67	8.91	0.46	12.31	4.29	25.66**	17.84**	24.60**	3.28	75.07**	29.20**	53.58*	-1.62
14.	Tift23AxPPMI493	-3.82**	1.34	31.63**	24.64**	107.41**	100.00**	35.68**	16.75**	7.14	-8.25	112.20**	61.40**	104.85**	42.07*
15.	Tift23AxJ254	5.63**	11.11**	27.64**	21.08**	31.03	26.67	30.19**	27.56**	9.72	-1.93	98.49**	80.14**	147.47**	108.51**
16.	5141AxD23	7.99**	19.86**	30.60**	21.23**	76.30**	58.67**	45.86**	36.20**	20.04**	-0.05	12.92	-17.48	156.74**	134.00**

Contd…

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No.	Cross														
17	5141AxICMP451	8.41**	16.78**	46.14**	41.24**	-10.34	-13.33	30.67**	21.33**	6.55	-12.01*	36.08**	3.13	30.49	23.60
18	5141AxPPMI301	-7.69**	-1.96	23.83**	21.00**	11.11	0.00	11.42*	8.35	14.8**	-6.02	24.61	-4.80	62.56**	23.38
19	5141AxPPMI493	-2.67*	-0.61	26.62**	26.32**	28.57	20.00	20.73**	7.35	15.23**	-2.70	35.00**	6.58	104.55**	73.08**
20	5141AxJ254	7.49**	22.22**	28.00**	14.83*	36.00	36.00	38.82*	31.14**	4.70	-7.72	46.76**	39.73*	121.80**	101.39**
21	841AxD23	-7.53**	-4.26*	15.79**	1.56	15.38	7.14	29.01**	12.57	7.92	-7.68	50.94**	25.87**	76.99**	71.43**
22	841AxICMP451	10.00**	10.74**	32.98**	29.23**	0.00	0.00	21.47**	21.33*	7.91	-7.81	22.60*	7.03	78.15**	76.52**
23	841AxPPMI301	1.32	1.99	13.65*	4.57	15.38	7.14	15.64**	10.39	18.13**	0.00	63.24**	44.00**	65.16**	31.20*
24	841AxPPMI493	-5.70**	-1.32	35.55*	28.02**	11.11	7.14	18.62**	13.04**	15.61**	1.11	27.92*	17.54	26.08	12.74
25	841AxJ254	6.99**	13.33**	28.57**	22.28**	44.83*	40.00	23.33**	8.78	12.86*	3.16	47.18**	29.84*	99.29**	71.04**
26	393AxD23	7.64**	14.89**	9.36	-9.02	-5.88	-27.27	23.06**	12.71*	13.21**	9.04	25.40**	10.49	75.52**	68.00**
27	393AxICMP451	-1.62	2.01	27.73**	16.93**	0.00	-80.18	14.25*	8.18	12.88**	8.56	55.27**	43.75**	73.83**	73.20**
28	393AxPPMI301	4.79**	7.19**	35.08**	17.50**	-11.76	-31.82	-1.41	-2.14	25.62**	19.53**	40.17**	31.20**	71.00**	34.71*
29	393AxPPMI493	-10.77**	-9.38**	37.67**	22.71**	2.86	-18.18	11.34*	0.85	11.16*	9.84	54.71**	51.32**	87.77**	66.11**
30	393AxJ254	-1.02	8.15**	43.29**	41.57**	18.92	0.00	30.63**	21.02**	29.45**	24.72**	64.29**	37.16**	189.37**	150.94**
31	267AxD23	11.40**	21.28**	30.01**	10.25*	41.18*	9.09	34.90**	30.67**	27.91**	6.63	47.45**	5.94	118.33**	87.14**
32	267AxICMP451	-2.86*	2.68	37.06**	28.21**	20.00	-1.82	13.53*	1.79	19.93**	-0.15	44.88**	7.81	125.52**	100.31**
33	267AxPPMI301	-7.21**	-3.27	21.40**	7.76	23.53	-4.55	11.01*	4.04	25.27**	3.38	29.07*	-3.20	14.39	-17.09
34	267AxPPMI493	2.72*	3.03	41.72**	28.99**	8.57	-13.64	8.62	-6.57	23.39**	5.08	51.27**	17.11	124.32**	79.57**
35	267AxJ254	4.32	16.30	39.73**	38.16**	8.11	-9.09	26.80**	24.33**	16.29**	3.33	57.93**	46.58**	100.10**	94.40**

*Significant at 5% level

**Significant at 1% level

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from 0.81 to 0.50 and cluster analysis was performed (Table 4). The dendrogram generated 4 clusters as shown in Figure 1. The three clusters (D23 and Tift23A; ICMP451, PPMI301, 863A and 267A; PPMI493, J254and 393A) joined at a similarity value of 68%. The fourth cluster (81A, 5141A and 841A) joined with the remaining at 61% similarity value

Correlation of genetic distance with hybrid performance and heterosis

Table 5 depicts the correlations of genetic distance with hybrid performance and heterosis over midparent and better parent in the pearl millet line and tester analysis. The correlation values mostly were not significant with genetic distance except for days to 50% flowering. For ear length and ear width, heterosis over midparent and better parent were significantly correlated. 1000 grain weight was not correlated with hybrid performance and heterosis.

DISCUSSION

The analysis of variance in pearl millet indicated that the parents were diverse for all the seven characters studied. Hallauer and Miranda (1988) and Smith (1986) have reported poor relationship between per se performance of lines and hybrid combinations. However, in our study, the per se performance of both CMS lines and restorer lines were significantly correlated with hybrid performance for three characters viz. days to 50% flowering, ear width and 1000 grain weight. Per se performance of hybrids indicated that many crosses were superior than the check Pusa 23 (data not shown). The grain yield of pearl millet is influenced by many component characters such as 1000 grain weight, ear length, ear width, productive tillers, plant height and days to flowering. The degree of influence of one variable on the other can be expressed in quantitative terms. Path Co-efficient analysis, a method employed by Dewey and Lu (1959) in plants, was very efficient in this respect. The result of path analysis revealed that productive tillers, ear width and days

Table 4 Genetic similarity values among seven CMS and five restorer lines used in the present study

	81A	863A	Tift23A	5141A	841A	393A	267A	D23	ICMP 451	PPMI 301	PPMI 493	J254
81A	1.00											
863A	0.57	1.00										
Tift23A	0.70	0.54	1.00									
5141A	0.82	0.57	0.79	1.00								
841A	0.66	0.68	0.54	0.61	1.00							
393A	0.70	0.64	0.59	0.70	0.50	1.00						
267A	0.59	0.57	0.57	0.59	0.57	0.61	1.00					
D23	0.61	0.54	0.73	0.57	0.54	0.64	0.66	1.00				
ICMP451	0.61	0.73	0.64	0.61	0.68	0.68	0.70	0.68	1.00			
PPMI301	0.57	0.59	0.64	0.66	0.68	0.68	0.52	0.64	0.73	1.00		
PPMI493	0.66	0.68	0.73	0.66	0.59	0.73	0.57	0.68	0.59	0.59	1.00	
J254	0.70	0.59	0.73	0.70	0.59	0.68	0.61	0.68	0.64	0.68	0.73	1.00

Figure 1   Genetic relationships among lines and testers of pearl millet used in the present study
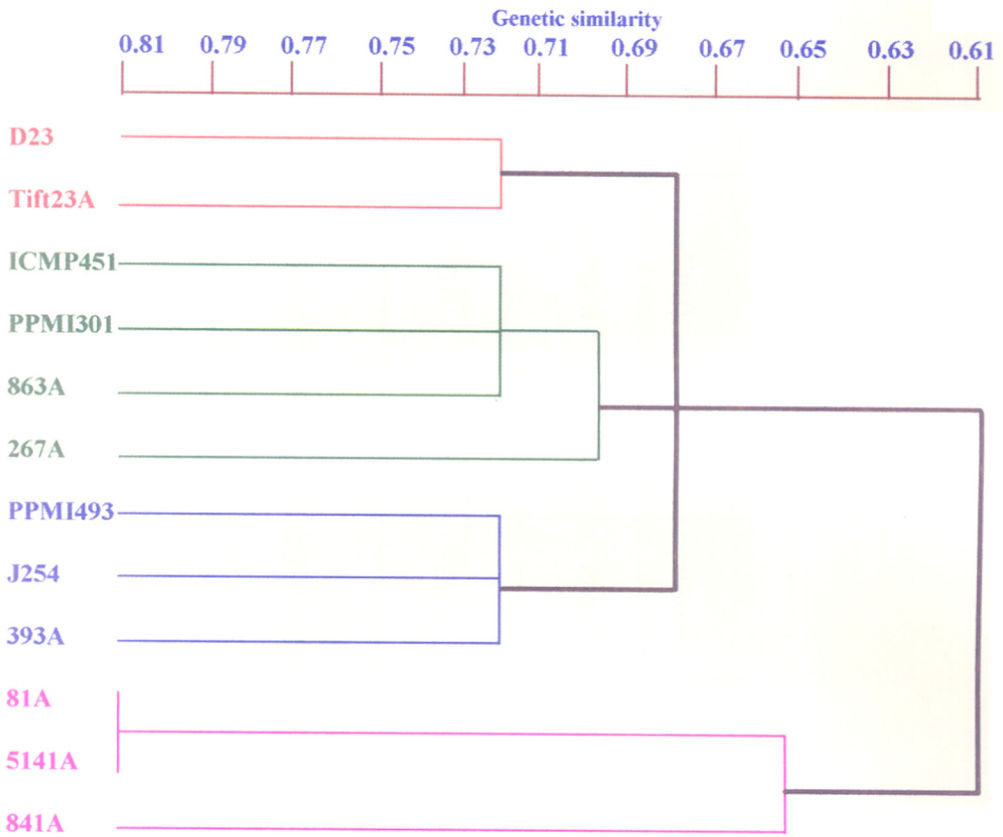
Table 5 Correlations of genetic distance (GD) with hybrid performance, heterosis over midparent (MPH) and better parent heterosis (BPH) in the pearl millet line and tester sets.

	Days to 50% flowering	Plant height	Productive tillers	Ear length	Ear width	100 grain weight	Grain yield per plot
GD with hybrid performance	0.223	-0.082	0.197	-0.027	0.025	-0.136	0.019
GD with heterosis over midparent	0.209*	0.125	0.062	0.326**	0.434***	0.056	0.089
GD with heterosis over better parent	0.275*	-0.090	-0.057	0.254*	0.362**	-0.046	0.194

* Significance at more than 5% level

** Significance at 5% level

***Significance at 1% level

to 50% flowering were very important component characters since they had relatively large positive direct effect on yield (data not shown).

In the present study, based on molecular analysis, all lines and testers grouped into four clusters and the relationship of inter and intra cluster genotypes with hybrid performance and heterosis for all the traits gave varied results. Even the field data for crosses gave similar information. Our results indicated that days to flowering trait showed significant correlation for hybrid performance and heterosis whereas significant association was found with ear length and ear width for heterosis.

The heterosis phenomenon was reported by many authors positively or negatively for various traits in pearl millet. Positive heterosis for grain yield, plant height, tiller number, ear length, ear girth, harvest index and for forage traits was reported by Soundarpandian *et al* 1964; Phul *et al* 1973; Subramaniam and Rathiram 1980, Bajaj and Phul 1982, Burton 1982 and Rao *et al* 1983. Negative heterosis for days to earing was found by Ahluwalia and Patnaik (1963). Our results showed very low levels of correlations and were not useful for predicting F1 performance, however, the information could be used for grouping and differentiating genotypes.

The exploitation of heterosis by breeding hybrid varieties offers a considerable scope for the improvement of pearl millet crop. Specific marker heterozygosity studies showed highly significant correlations in rice (Xiao *et al* 1996b). However, there are very few markers so far reported to be associated with QTLs for yield in pearl millet.

In summary, the microsatellite (GATA)$_4$ which is highly polymorphic alongwith RAPDs can be used for grouping of parents and for identifying specific markers linked to such QTLs. Predictions based on specific markers may help in developing promising hybrids in pearl milllet crop.

CHAPTER IV

THESIS OVERVIEW AND FUTURE

PERSPECTIVES

Present status of rice and pearl millet breeding program in India

Rice and pearl millet are important food crops occupying first and fourth position, respectively, among cereals, in India. Rice is one of the main staple food for a large segment of the Indian population while pearl millet is considered to be the 'poor man's crop'. With the green revolution, there has been a significant impact on the production of food grains in India. According to the statistical figures, by the end of the 20th century, the Indian population will almost reach the 1billion mark. An urgency is, therefore, felt to increase the present production levels of food grains, in order to meet the ever increasing demand for these crops.

Though a sustainable production of rice has been achieved, so far, there is a plateau in the production rate due to the consistent cultivation of a few cultivars which fare well at more than one location. Pearl millet is one of the most drought tolerant crops among cereals and can be grown in the regions where wheat and rice fail to grow. However, the production of pearl millet is hampered due to low hybrid coverage, slow varietal spread and diseases.

Genetic diversity studies in rice and pearl millet using DNA markers

To cope up with the demands of the increasing population, a need is now felt to combine the efforts of the breeders and of molecular biologists working in the laboratories, so as to bring about a 'molecular green revolution' in the near future. A combination of marker assisted selection and genetic engineering has definitely facilitated the development of a variety of crop plants which are high yielding and also highly resistant to biotic and abiotic stresses.

Genetic variation in the germplasm can be a basis for the improvement of plant varieties. Development of superior varieties through hybrid breeding programs is an important aspect of plant breeding. The development of DNA markers such as RFLPs, repetitive DNA markers and PCR-based DNA

markers, have made the selection of plant types much easier and rapid compared to conventional breeding practices. Microsatellites and minisatellites have been shown to be abundant and highly polymorphic DNA markers. Microsatellite markers based on simple sequence repeats (SSRs) have been developed in many crop species, including rice, barley, grapevine, brssica, maize, soybean and tomato. Simple sequence length polymorphisms (SSLPs) have already been demonstrated to be a powerful tool in genotype identification for variety protection, seed purity evaluation, germplasm conservation, diversity studies, gene and quantitative trait loci (QTL) analysis, pedigree analysis and marker assisted breeding. Apart from this, PCR-based markers such as RAPDs are shown to have good potential for saturation of the genetic linkage maps.

Rice is one of the most polymorphic crop species, being composed of diverse ecological groups referred to as subspecies. The habitats of these subspecies range from tropical to subtropical to temperate zones (Oka 1988). The cultivated rice gene pool consists of a wealth of landraces, improved varieties and an increasingly large number of lines for hybrid rice production. The RAPDs alongwith pedigree data have shown very limited genetic variability in the Indian elite rice cultivars. This aspect needs to be considered while developing future elite varieties. Wide range of variation is also observed for several characters in pearl millet germplasm, consisting of landraces and wilds.

In our pearl millet studies, various simple sequence repeats have been characterized for their abundance and polymorphism in cultivars and landraces. Tetranucleotide repeat $(GATA)_4$ has been found to be abundant in the genome and has proved extremely useful as a DNA fingerprinting probe. The present data on microsatellites in pearl millet can be further used to develop saturated linkage maps. Randomly amplified polymorphic DNAs (RAPDs) have shown to be useful in genetic fingerprinting and parentage analysis in pearl millet. Classification of pearl millet cultivars and landraces based on RAPDs is not same as compared to microsatellite markers. This

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indicates that both these classes of markers originate from different loci in the genome.

Application of DNA markers in prediction of heterotic combinations in hybrid programs

Prediction of hybrid performance has been of interest for long. The advantages of DNA markers have made them extremely popular in recent years for predicting hybrid performance and heterosis in crop breeding programs. This can accelerate the development of new hybrids in various crops in a short span of time. A number of reviews and research articles have been published in recent years regarding the application of DNA markers in plant genome analysis and plant breeding programs. Based on the genetic diversity between two parents, several attempts have been made to date, so as to predict the correlation between molecular heterozygosity and heterosis. There are several controversial reports for prediction of heterotic combinations based on genetic diversity. With the quantitative trait loci being identified for traits which affect the heterosis phenomenon, it may now be possible to identify markers linked to traits, which are responsible for bringing positive heterosis. The development of such markers will definitely refine the process, which though in its infancy today, is thought to be the method for predicting heterosis before actually carrying out the crosses, in future. Large number of studies in corn indicate that the relationship between marker based genotype divergence among parents and hybrid performance is variable. The studies on rice and pearl millet have shown that molecular heteozygosity determined by RAPD markers does not show the correlation with hybrid performance.

Future perspectives

Allelic diversity studies can be carried out in Indian elite rice cultivars using primers flanking the microsatellites by PCR amplification. In rice and pearl millet, use of various classes of DNA markers and more number of

crosses involving various cytoplasmic male sterile lines and restorer lines can lead to use of these markers in prediction of heterotic combinations. Finally, identification of marker loci which are significantly associated with traits of interest and unraveling of complex genetic interactions (epistasis) involved in heterosis phenomenon, will revolutionize the new marker based hybrid programs.

The genetic linkage map of pearl millet is not quite saturated so far. The genome analysis of pearl millet has indicated the presence of various micro and minisatellite repeats, which can be utilized for the saturation of this map. These markers may be then useful for monitoring the gene introgression between wild and cultivars in a test cross. Also they could be ideally utilized for tagging economically important QTLs responsible for downy mildew resistance, yield, abiotic stresses, drought tolerance and phenomenon like apomixis.

THESIS ABSTRACT

Assessment of a genetic diversity was carried out in Indian rice elite cultivars and pearl millet varieties and pearl millet landraces using DNA markers such as microsatellites and RAPDs. Cytoplasmic Male Sterile and restorer lines which are being used in hybrid breeding programs were selected in rice and pearl millet and the genetic distance levels were correlated with field data for hybrid performance and heterosis. The highlights of various findings are summarized as follows :

Genetic diversity evaluation of Indian elite rice varieties using RAPD markers

RAPD markers were used to evaluate the genetic diversity in 44 Indian elite rice cultivars. Out of 40 operon primers, eight primers (OPA1, OPA7, OPA12, OPA13, OPA17, OPA18, OPB7 and OPB18) amplified 40 reproducible fragments and 80% of them showed polymorphism.

The similarity data grouped the 44 cultivars into two clusters with a similarity ranging from 60% to 90% with the exception of Bhavani and Jal Manga cultivars having 46% similarity with the remaining cultivars. The results indicate that at the smaller subgroup level,. the elite rice cultivars are genetically more similar.

A very limited genetic variability has been observed in elite cultivars using RAPD markers and there is a need to broaden the genetic base in developing Indian elite rice varieties in the future.

Use of DNA markers in prediction of hybrid performance and heterosis in rice

Out of 46 operon primers attempted on a total of 18 rice genotypes including two CMS lines and 16 restorers lines, 35 primers generated 137 polymorphic products.

Based on RAPD data, cluster analysis was performed and the dendrogram generated 4 clusters, in which a CMS line IR58025A was closer to two clusters, whereas CMS line IR62829A was closer to one cluster only.

The genetic distance was negatively correlated with hybrid performance , heterosis over midparent and heterosis over better parent for plant height, percentage of productive tillers and grain yield per plant. Many traits showed no association with genetic distance.

There is more significant negative correlation with IR 62829A compared to IR 58025A, with respect to tillers and productive tillers per plant

Genotype identification and assessment of genetic relationship in pearl millet (*Pennisetum glaucum* L.) using (GATA)$_4$ microsatellite and RAPDs

The potential of DNA markers such as microsatellites, minisatellites and RAPDs was investigated in pearl millet (*Pennisetum glaucum* L.) with respect to their abundance and variability.

Southern analysis using twenty two different di-,tri-,tetra-and penta-oligonucleotide probes and five minisatellite probes identified (GATA)$_4$ as the most useful probe for the detection of multiple polymorphic fragments among pearl millet cultivars and landraces from India.

The clustering patterns of pearl millet cultivars and landraces based on (GATA)$_4$ and RAPD markers differed. The landraces representing eight states in India could not be grouped based on their geographic distribution with DNA markers.

The probability of identical match by chance for any two genotypes using (GATA)$_4$ and RAPDs was 3.02×10^{-24} for cultivars and 0.52×10^{-12} in case of landraces.

The microsatellite (GATA)$_4$ and RAPDs have offered useful tools for genotype identification and assessment of genetic relationship in pearl millet.

Hybrid performance and genetic distance as revealed by (GATA)$_4$ microsatellite and RAPD markers in pearl millet

Genetic diversity in five cytoplasmic male sterile and seven restorer lines of pearl millet was determined by DNA fingerprinting using (GATA)$_4$ microsatellite and RAPDs.

A total of 160 polymorphic loci were generated and based on the polymorphism data, cluster analysis was performed. The genetic relationships among these lines revealed that they were not in agreement with the available pedigree data.

Per se performance of parents and hybrids was analyzed for days to 50% flowering, plant height, productive tillers, ear length, ear width, 1000 grain weight and grain yield per plot.

Path co-efficient analysis revealed that productive tillers, ear width and days to 50% flowering had relatively large positive effect.

The correlation values mostly were not significant with genetic distance except for days to 50% flowering, ear length and ear width.

Results have indicated that genetic distance measures based on $(GATA)_4$ microsatellite and RAPDs may be useful for grouping of parents but not for predicting heterotic combinations in pearl millet.

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Publications

1. **Chowdari KV**, Davierwala AP, Govila OP, Gupta VS, Ranjekar PK.(1998) Genotype identification and assessment of genetic relationship in pearl millet (Pennisetum glaucum L.) using $(GATA)_4$ microsatellite and RAPDs. Theor Appl Genet (In press).

2. **Chowdari KV**, Venkatachalam S, Davierwala AP, Govila OP, Gupta VS, Ranjekar PK. (1998) Hybrid performance and genetic distance as revealed by $(GATA)_4$ microsatellite and RAPD markers in pearl millet. (Accepted for publication in Theor Appl Genet).

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