GENETIC DIVERSITY ANALYSIS IN DOUBLED HAPLOIDS OF RICE USING MOLECULAR MARKERS

Thesis submitted in part fulfilment of the requirement for the degree of ***Master of Science in Biotechnology*** *to the Tamil Nadu Agricultural University, Coimbatore.*

By

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CERTIFICATE

This is to certify that the thesis entitled **"GENETIC DIVERSITY ANALYSIS IN DOUBLED HAPLOIDS OF RICE USING MOLECULAR MARKERS"** submitted in part fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Ms.RAKHI RAJAN**, under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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ABSTRACT

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A study was conducted to assess the genetic diversity present among the doubled haploids (DH) synthesized *in vitro* from the anthers of the hybrids of TNRH16 (IR58025A x C20R), TNRH19 (IR58025Ax CR1009) and TNRH21 (IR58025A x IR21567R) along with the male fertile line IR58025B. A total of 14 DH lines, three hybrids and their five respective parents were used for the analysis involving four isozyme systems and 30 random decamer primers.

Isozyme analysis was carried out using four enzyme systems – esterase, super oxide dismutase (SOD), polyphenol oxidase (PPO) and peroxidase. Of these four enzymes, esterase and SOD were monomorphic among the DH lines. In the case of PPO, the absence of lower bands were noticed in the case of the genotypes TNRH16/30-6, TNRH16/30-7, TNRH19/8-55, TNRH21, TNRH21/6-60 and TNRH21/8-70 and in peroxidase the lower band was absent in three lines *viz.*, TNRH19/1-37, TNRH19/8-55 and IR58025B. However, these four markers were considered to be artifacts rather than markers since all the bands were present in the respective parents from which these hybrids were derived. Using this data,

dendrogram was constructed and the similarity values showed that all the DH lines were atleast 89 per cent related to each other.

RAPD analysis was carried out using 30 decamer random primers and all of them amplified scorable products. A total of 280 markers were produced. Of these 87 were found to be polymorphic. Out of the 30 primers used, OPF10 was found to produce markers which can differentiate TNRH16 and TNRH16 anther derived DH lines. This primer also revealed the relatedness of the hybrid with its male parent C20R. The cluster analysis based on band sharing data obtained by RAPD analysis grouped the DH lines of the three hybrids into three different clusters. The marker analysis established the similarity between IR58025A and IR58025B. The other two parents *viz.*, CR1009 and C20R were separated from the other genotypes.

From the present study, it is clear that RAPD markers can be effectively used for genetic diversity analysis in rice over the isozyme markers.

CONTENTS

ABBREVIATIONS

AFLP	-	Amplified Fragment Length Polymorphism
AP-PCR	-	Arbitrarily Primed Polymerase Chain Reaction
APS	-	Ammonium Per Sulphate
bp	-	base pair
BPB	-	Bromophenol blue
cDNA	-	complementary DNA
cM	-	centi Morgan
CMS	-	Cytoplasmic Male Sterile
CPMB	-	Centre for Plant Molecular Biology
CTAB	-	Cetyl or hexadecyl trimethyl ammonium bromide
DH	-	Doubled Haploid
dNTP	-	deoxy nucleotide triphosphate
DNA	-	Deoxy ribonucleic acid
EDTA	-	Ethylene diamine tetra acetic acid
Est	-	Esterase
F_1	-	Fisrt Filial generation
F_2	-	Second Filial genaration
FISH	-	Flourescent *In situ* Hybridization
IR21567R	-	IR21567-22-19-3R
ISSR-PCR	-	Inter Simple Sequence Repeat PCR
Kbp	-	kilo base pair
KDa	-	kilo Dalton
NIL	-	Near Isogeneic Lines
NTSYS	-	Numerical Taxonomy and multivariate analysis system
OP	-	Operon
PAGE	-	Polyacrylamide Gel Electrophoresis
PBS	-	Paddy Breeding Station
PCR	-	Polymerase Chain Reaction

PPO	-	Polyphenol oxidase
Prx	-	Peroxidase
QTL	-	Quantitative Trait Locus
RAPD	-	Random Amplified Polymorphic DNA
RFLP	-	Restriction Fragment Length Polymorphism
RGP	-	Rice Genome Project
Rm	-	Relative mobility
SCAR	-	Sequence Characterized Amplified Regions
SDS	-	Sodium Dodecyl Sulphate
SGE	-	Starch Gel Electrophoresis
SI	-	Similarity Index
SOD	-	Super oxide dismutase
SSR	-	Simple Sequence Repeats
STMS	-	Sequence Tagged Microsatellite Sites
STS	-	Sequence Tagged Sites
TAS	-	Telomeric repetitive Associated Sequence
TBE	-	Tris Borate EDTA
TE	-	Tris EDTA
TEMED	-	N,N,N,N, tetramethyl ethylene diamine
TNAU	-	Tamil Nadu Agricultural University
TNE	-	Tris NaCl(Sodium chloride) EDTA
TNRH	-	Tamil Nadu Rice Hybrid
TRIS	-	Tris (hydroxymethyl) aminomethane
UPGMA	-	Unweighted Paired Group Method with Average mean
uv	-	ultraviolet
VNTR	-	Variable Number of Tandem Repeats
WA	-	Wild Abortive

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INTRODUCTION

CHAPTER I
INTRODUCTION

Rice contributes around 45 per cent to India's total cereal production. The minimum production and productivity required for rice has been estimated (on the basis of population growth at 1.9 per cent and income growth at 5 per cent) to be 100 million tonnes and 2,454 kg/ha respectively by 2006-2007 as against the present 82 million tonnes and 1,851 kg/ha. This amounts to a minimum growth requirement of 2.3 per cent (Siddiq, 1999). One of the ways for increasing the rice productivity is by exploiting the potentials of heterosis breeding. India earned the distinction of emerging as the second country after China to exploit hybrid vigour in rice when it successfully developed and released for commercial cultivation the first set of five hybrids in 1994. Since then, the area under hybrid rice increased to over 1.5 lakh ha. But the problem in exploiting the heterosis breeding is the loss of hybrid vigour after F_1 generation resulting in yield reduction. This necessitates the synthesis of fresh F_1 seeds for each season. The other possible alternative to fix the heterosis is by synthesizing doubled haploids (DH) from the pollen of F_1 hybrids. Anther culture allows the production of homozygous plants in two generations and thus reducing the time required to produce new rice varieties with increased yield. It also enables greater utilization of minor genes for resistance, the cumulative effect of which may provide more durable resistance than that provided by major genes. It has been known for more than 20 years that phenotypic variation occurs in plants regenerated from cultured anthers. Commonly observed variation in anther culture derived plants includes the number of tillers per plant, plant height, flag leaf length, heading date, panicle length, fertility, and the number of seeds produced (Oono, 1985; Sun *et al.*, 1983). Molecular markers like isozymes, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), microsatellite markers, *etc.* can be used as tools to check whether the variations are present in the DH lines. Marker analysis of DH lines will pave the way to understand the genetic makeup of the DH lines. Ultimately, the phenotypic variations observed

can be correlated to the molecular marker profile of each and every line if polymorphism is seen.

In the present study, fourteen doubled haploid plants derived from the anthers of three Tamil Nadu rice hybrids, the three respective hybrids and their parents were used to assess the diversity involving isozyme and RAPD markers. The major objectives of the study are:

- Assessing the genetic diversity of DH lines along with their respective hybrids and parents using isozyme and RAPD markers.
- Assessing the differences between DH lines of individual hybrids
- Comparing the efficiency of marker system in genetic diversity analysis by involving the above mentioned genetic materials.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Rice is the staple food of Indians. The population of India is increasing alarmingly and to feed this increasing population our food production should also increase. It is not possible to increase production by increasing the land area under cultivation as it is almost saturated. The next alternative is to increase the productivity. For achieving this, high yielding varieties play an important role. One of the methods of producing high yielding varieties, is by breeding utilising the hybrid vigour. But the problem encountered here is the loss of hybrid vigour in succeeding generations. In this context, the scope of anther culture is very vast. The anthers of hybrids if used for propagation, hybrid vigour can be fixed. The gametic genotype, including recessive genes, is expressed and early generation selection becomes feasible due to the additive effect of the doubled haploid lines and the elimination of dominance variance. It also enables greater utilization of minor genes for resistance, the cumulative effect of which may provide more durable resistance than that provided by major genes. (Toenniessen and Khush, 1991).

One of the most crucial concerns in *in vitro* propagation is to retain genomic integrity of the micropropagated plants *vis-a-vis* mother plant(s) so that the advantages (high yield, uniform quality of commercial product, shorter rotation period *etc.*) in the use of elite genotype(s) over natural seedlings are maintained. Anther culture is also done for producing offspring which are true-to-type to the mother plant. But somaclonal variation is common and also variation can occur between different microspore derived plants. Thus genetic diversity exist between doubled haploids also. The genetic diversity has to be assessed correctly for any breeding program. It can be done by morphological or molecular analyses.

2.1. Analysis based on morphological traits

The use of *ex situ* conserved germplasm and the efficient management of collections by breeders depend, to a large extent, on the availability of information on the genetic diversity which resides in the collections. The process of gathering this

information is commonly referred to as characterization and evaluation : accessions are grown under suitable conditions and data are recorded for a number of descriptors, which may be defined as identifiable and measurable plant attributes. Characterization descriptors, such as flower colour, are highly heritable, easily seen by eye and equally expressed in all environments. They allow rapid discrimination of phenotypes. Evaluation descriptors, such as yield and disease resistance, are under polygenic control and susceptible to environmental influence but are important in crop improvement. Description of the morphology of vegetative and reproductive organs and classical agronomic assessment has been the main stay of genetic resources characterization and evaluation before the development of molecular markers for this purpose.

Polymorphic, highly heritable morphological traits were some of the earliest genetic markers employed in scientific investigation (De Vries, 1912). Morphological assays generally require neither sophisticated equipments nor preparatory procedures. So monogenic or oligogenic morphological traits are generally simple, rapid, inexpensive to score, and even from preserved specimens. Some of these morphological characters served as genetic markers (Gottlieb,1984; Hilu,1984) suitable for plant germplasm management. The lower heritability and substantial genotype versus environmental interactions dramatically increase the complexity and expense of assaying the polygenic morphological traits (Patterson and Weatherup, 1984). Furthermore, determining homology from analogy may be more costly and complicated than with other genetic markers (Camussi *et al.*, 1985) Since these morphological markers are often influenced by environment, which require special breeding program and experimental design to distinguish genotypic and phenotypic variation in a population, they are replaced by molecular markers. Until recently, scientific plant classification was based nearly exclusively on morphological assays (Stuessy, 1990).

2.2 Molecular markers

In recent years, new molecular genetic techniques have increasingly been used to describe plant germplasm collections (Rao and Riley, 1994). The advantages of molecular markers for germplasm characterization are that any part of a plant can be used, only small amounts of tissue are required from which the whole genome can be assayed, generally the method of inheritance is simple, and growth conditions have no effect on expression. There are mainly two types of markers-biochemical markers like isozymes and proteins and DNA based markers like Restriction Fragment Length Polymorphism (RFLP) and PCR based markers such as Randomly Amplified Polymorphic DNAs (RAPDs), SSR (Small Sequence Repeats) or microsatellites, Sequence Tagged Sites (STS), Sequence Characterized Amplified Region (SCARs), Variable Number Tandem Repeats (VNTRs or minisatellites,), and Amplified Fragment Length Polymorphism (AFLP).

The relative advantages of molecular markers over morphological markers for most genetic and breeding applications were discussed by Tanksley *et al.* (1989) and Stuber (1992). The significance of molecular markers in rice systematics and evaluation of genetic resources had been reported by Second (1991). The different molecular marker technologies for plant improvement have also been reviewed by Winter and Kahl (1995). Molecular markers can be used for different purposes like genetic diversity analysis, construction of genome maps, location of a particular desirable gene in a chromosome, determination of quantitative trait loci *etc.* Some of the important works using molecular markers under these areas are reviewed here.

The applications of molecular markers to assess plant genetic diversity was studied by Briquet *et al.* (1996). An alternative way to predict quantitative variation with rice germplasm using molecular markers had been reported by Virk *et al.*(1996). Linkages between the different marker types (RFLP, isozyme and morphological markers) in rice had been studied by Mishra *et al.* (1996). The different marker types revealing contrasting genetic diversity relationships in rice had been reported by Parsons *et al.* (1997). *Oryza sativa* L. had been successfully characterized genetically based on morphology, isozyme and RAPD markers by Sun *et al.* (1997).

The potential use of molecular markers in the management of genetic resources in seed gene banks was discussed as a case study in rice by Ford-Llyod *et al.* (1997).

2.2.1. Isozyme markers

During the last 20 years isozymes revealed through starch gel electrophoresis (SGE) have been the genetic markers most frequently employed for plant germplasm management. The intervarietal variation and classification of cultivated rice were studied by Oka (1958). Isozyme studies in rice were initiated in mid 1960's. The esterase isozyme loci of *Oryza sativa* L. *i.e*; Est-2 on chromosome 6 in rice was detected by Nakagahra and Hayashi (1976). Pai and Fu (1977) performed genetic analysis for peroxidase and acid phosphate isozymes in cultivated rice.

Isozymes are useful as genetic markers since they are co-dominant and also exhibit low level of environmental interactions (Gottlieb, 1981). Therefore, isozymes provide a cheap and effective method for classifying the different varieties/populations according to their genetic composition. Furthermore, characterizing germplasm on the basis of both isozymes and morpho/physiological characters would help to eliminate duplications of germplasm collections.

A varietal classification of Asian cultivated rice (*Oryza sativa* L.) based on isozyme polymorphisms was reported by Glaszmann (1985). Evolutionary relationships in the *sativa* group of *Oryza* could be inferred based on isozyme data as reported by Second (1985). To classify rice germplasm, fifteen polymorphic isozyme loci were used to group 1688 rice accessions from Asia by Glaszmann (1987). The accessions were grouped into six distinct groups and now this procedure is routinely used to classify germplasm in all other crops.

Glaszmann *et al.* (1988) reported the electrophoretic variation of isozymes in plumules of rice which functioned as a key to the identification of 76 alleles at 24 loci. The chromosomal locations of 10 isozymes loci in rice was found out by Wu *et al.* (1988) through trisomic analysis. The applications of isozymes to crop evolution had been examined by Doebley (1989). Pham *et al.* (1990) have discussed the genetic

analysis and linkage relationships of different isozyme markers in rice. Specificity of expression of esterase isozyme genes in rice was studied by Cai *et al.* (1992).

Ishikawa *et al.* (1992) studied the genotypic variation for isozyme genes among Japanese upland varieties in rice. Romero *et al.* (1993) conducted comparative studies on isozyme in *Oryza sativa, O.minuta* and their interspecific derivatives for ten isozyme system. It was observed that between the two species, two systems were monomorphic (isocitrate dehydrogenase and alcohol dehydrogenase) and eight were polymorphic which indicates the genotypic variation.

A simple way to identify distant hybrids with the help of characteristic bands of esterase isozyme in the leaves of rice (*Oryza*) was reported by Xu and Xian (1995). Sun Xinhi *et al.* (1996) reported a simple and efficient method for studying electrophoretic variation of isozymes in rice with polyacrylamide gels. Lee *et al.* (1996) successfully classified Korean rice germplasm based on isozyme polymorphism. This will be useful in locating the genes. Protein polymorphism of barley cultivars was studied through superoxide dismutase (SOD) isoenzyme analysis by Zvingila *et al.* (1998). SOD isozyme spectra from roots, grains and leaves of 12 barley cultivars were established. Variability between cultivars and changes of isozyme activity during organogenesis were detected by this method. In spite of changes in SOD banding pattern in seeds was constant and can be used to study the genetic variability among barley cultivars. A high density genetic linkage map comprising of 444 loci was constructed from the doubled haploid population derived from an inter-subspecific cross between an *Oryza sativa* ssp *indica* variety Zhaiyeqing 8 and *japonica* variety Jingxi 17 using 9 isozymes, RFLP and RAPD markers (Li Shuang *et al.*, 1999).

2.2.2. DNA markers

The limitations encountered in the protein and isozyme markers namely the low level of polymorphism and failure to express the polymorphism due to base substitution could be overcome by the use of DNA markers. The markers at DNA level include short DNA sequence, whole genes or even longer segments of DNA.

The detection and exploitation of naturally occurring DNA sequence polymorphisms represent one of the most significant developments in molecular biology. Restriction endonuclease digestion of total genomic DNA followed by hybridization with a radioactively labeled probe reveals differently sized hybridizing fragments. This form of polymorphisms termed Restriction Fragment Length Polymorphism (RFLP) has been used extensively for genetic studies. But technical complexity of RFLP coupled with the problem of short lived radioisotopes are limitations of this methods.

Since its development, the Polymerase Chain Reaction (PCR) has revolutionized many standard molecular biological techniques, with modifications of the original procedure designed to suit a range of needs. One such variation generates a specific class of molecular markers termed Randomly Amplified Polymorphic DNA (RAPDs). RAPDs are well suited for use in the large sample throughout systems required for plant breeding, population genetics and studies of biodiversity.

2.2.2.1. RFLP

RFLP markers have proven to be a reliable and highly informative tool for characterizing genetic diversity. Joint analysis of inbred lines and populations should provide valuable information with respect to

1. a better understanding of the genetic basis of present elite germplasm, and
2. the identification of populations that may prove to be useful sources of genetic diversity for breeding programmes.

Among the various molecular markers developed, RFLPs were the first to be used in human genome mapping and later they were adopted for plant genome mapping. Original documentation of RFLPs came from the findings of Grodzicker *et al.* (1974). RFLPs are codominant markers, inherited in a simple Mendelian fashion (Botstein *et al.*, 1980). RFLP is the most reliable polymorphism which can be used for accurate scoring of genotypes.

The homogenity of anther-culture derived lines of maize had been evaluated by means of field observations and molecular markers (Murigneux *et al.*, 1993). More than 100 RFLP markers had been tested on 189 DH lines. The study proved that the

heterogenity existing between the doubled haploids were very little. Genetic diversity analysis can be done using mitochondrial DNA also. Phylogenetic relationship in the genus *Oryza* had been done based on mitochondrial RFLPs (Abe *et al.*, 1999). The studies showed that even in *Oryza sativa*, the RFLP patterns of *japonica* and *indica* subspecies were clearly different from each other when 3 different probes were used.

Relationships among maize inbred lines and populations from European and North American origins had been estimated using RFLP markers (Dubreuil and Charcosset, 1999). Joint data analyses showed that the populations displayed a large number of alleles that were absent in the set of inbred lines. Associations among inbreds and populations further proved consistent with pedigree data of the inbreds and provided new information on the genetical basis of heterotic groups.

2.2.2.2. Polymerase Chain Reaction (PCR) based markers

The polymerase chain reaction is a recent revolution in the field of molecular biology (Mullis and Faloona, 1987). PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequence which uses two oligonucleotide primers of about 10-20 nucleotides in length that specifically hybridise to opposite strands flanking the region to be synthesized. Several cycles of DNA denaturation, primer annealing and extension of annealed primers by DNA polymerase, produce an exponential amplification of specific DNA sequences. The following are the different kinds of PCR based markers which are useful in various fields of molecular biology.

2.2.2.2.1. Sequence Tagged Sites (STS) Markers

Speed, efficiency and safety consideration have led many genome mapping projects to evaluate PCR sequence amplification as an alternative to RFLP. Informative primers are synthesized from known sequences, or end sequencing the anonymous clones to amplify the regions of interest. This approach is called Sequence Tagged Site (STS) approach (Tragoonrung *et al*, 1992). The use of STSs as standard landmarks in the rice genome has been reported by Minobe *et al.* (1994).

RAPD markers generated had been converted into Sequence Tagged Sites (STSs) and STS-specific primers in rice (Monna *et al*, 1994). Sixty random decamers were used both singly and in random pairs using DNA of a cross between *japonica* and *indica* varieties including the parents. Polymorphic bands, whose size varied from less than 100 bp to 2 kbp were obtained. Out of 102 RAPD markers, 20 STSs and STS-specific primers were determined by cloning, identifying and sequencing of the mapped polymorphic fragments.

2.2.2.2.2 Microsatellites or Small Sequence Repeats (SSR)

The genome of all eukaryotes appear to contain a special class of loci, termed microsatellites, which can serve, if sequenced and taken as the substrate for PCR, as highly informative, locus-specific markers. They are usually less than 100 bp long and are embedded in DNA with unique sequence. Genetic maps based on Sequence Tagged Microsatellite Sites (STMS) have the advantage that mapping vocabularies will be standardized to the DNA sequence base and that access to any particular locus will not require shipping and storing cloned probes. The species map will consist simply of a listing of nucleotide sequences. Reference populations for developing STMS maps can be chosen on the basis of biological or economic interest. It will not be necessary to maximize for genetic divergence. These and much more advantages had been listed by Beckmann and Soller (1990).

Microsatellites had been used to study the genetic difference between wheat cultivars (Devos *et al.*, 1995) using microsatellites in wheat storage proteins- one in a γ -gliadin pseudogene and one in a low molecular weight glutenin gene. Genetic diversity and its relationship to hybrid performance and heterosis in rice had been studied by PCR based markers (Xiao *et al.*, 1996). Ten elite inbred lines (4 *japonica* and 6 *indica*) were chosen and crossed to produce all possible hybrids excluding reciprocal. Analysis was done using RAPD and microsatellite. Results indicated that genetic distance measures based on RAPDs and SSRs may be useful for predicting yield potential and heterosis of intra-specific hybrids, but not inter subspecies hybrid.

Genetic molecular linkage map was constructed for a doubled haploid population derived from an inter-subspecific cross between an *indica* and *japonica* variety using 89 microsatellite markers along with RFLP, RAPD, AFLP, Telomeric repetitive Associated Sequence (TAS) and isozyme marker (Li Shuang and Lihuang, 1998).

The incorporation of heteroduplex analysis into conventional strategies for the study of polymorphisms at microsatellite loci has provided information useful in determining genetic diversity and relationships among organisms. (Perez, *et al.*, 1999). For testing this strategy, several *Solanum tuberosum* varieties had been checked using (TCT)n microsatellite located in intron I from the gene for granule-bound starch synthase. The data obtained confirmed the high degree of agreement between molecular and former taxonomy.

2.2.2.2.3 RAPD

A number of molecular techniques that are suitable for generating DNA profiles are currently available. A technique that is becoming particularly popular uses the PCR to generate Random Amplified Polymorphic DNA fragments (RAPDs) (Williams *et al.*, 1990). RAPD analysis can be performed on any organisms with no prior DNA sequence information. It is effective with tiny amounts of DNA. In an extreme example, Brown *et al.* (1993) showed that RAPD amplification was possible with DNA isolated from a single tobacco protoplast. The technology is relatively simple and cheap, allowing the analysis of a large number of samples in a short time.

The use of RAPDs for identification of rice accessions was suggested by Fukuoka *et al.*,(1992). RAPD analysis of 37 geographically diverse Australian rice varieities was performed by Ko *et al.* (1994). Three varieties from more distant geographical centres could be distinguished producing variety specific amplification products and a lower similarity index value compared to the rest of the varieties. Genetic variation between upland and lowland rice cultivars (*Oryza sativa* L.) could be detected by RAPD markers. Out of 42 random primers tested generating 260 amplification products, dendrogram constructed was identical to earlier classification based on isozyme markers reported by Yu and Nguyen (1994).

Polymorphism and genetic relatedness among wild and cultivated rice cultivars were studied by arbitrarily primed PCR (AP-PCR) analysis. The genetic distances revealed that cultivated rice (*Oryza sativa* L.) exhibited closest molecular affinity to wild rice (*Oryza rufipogon*) suggesting that origin of cultivated rice is from *Oryza rufipogon*. *Indica* and *japonica* subspecies showed closer affinity with *O.rufipogon* from different origins than with each other, supporting a multicentric origin of rice cultivation reported by Qingming *et al.* (1995).

The genetic polymorphisms of Korean red rice based on RAPD markers had been reported by Chan *et al.* (1995). Among the 24 strains of Korean red rice, one foreign red rice and five cultivars studied, two main groups could be differentiated, short grown red rices (*japonica* cultivars) and long grain red rices (*indica* and long type cultivars). RAPD marker had been successfully used for the study of diversity within plant germplasm collections of rice (Virk *et al.*, 1995). RAPD markers have been used to identify cultivated and wild rice species and had been reported by Farooq *et al.* (1995). Based on the RAPD polymorphisms developed, three out of five cultivated and five out of 11 wild rice entries were distinguished from each other. Inter and intraspecific variation were also detected. The marker identified in this study can be used to check seed quality, to fingerprint a specific entry and to detect transfer of genetic material from wild species to cultivated varieties.

The classification of cultivated rice (*Oryza sativa* L.) and common Chinese wild rice (*Oryza rufipogan*) was studied using RAPD by Chuan Qing *et al.* (1995). Primers revealed polymorphism between *indicia* and *japonica* varieties. The differences between *Oryza rufipogon* and *Oryza sativa* species occurred mainly between the wild rice and *indica* varieties with most accessions of wild rice having similar RAPD patterns to those of *japonica* accessions.

Diverse Asian rice (*Oryza sativa*) germplasm had been used to identify associations between various quantitative traits and RAPD markers using multiple regression analysis (Virk *et al*, 1996). This had allowed the prediction for other samples of germplasm about the performance for traits such as culm length and number of days for flowering, grain width and panicle and leaf length using only RAPD marker data.

The polymorphism of Chinese common wild rice (*O. rufipogon*) and cultivated rice (*Oryza sativa* L.) as determined by RAPDs have been reported by Huang *et al.* (1996). A dendrogram and a scattered figure demonstrating *indica-japonica* and wild-cultivated differentiations among the 90 rice accessions analysed were constructed based on RAPD data. The results indicated that Chinese common wild rice, *indica* rice and *japonica* rice could be clustered into three distinct groups.

The genetic diversity and subspecies differentiation of local rice cultivars from Manipur State of India had been reported by Kaneda *et al.* (1996). Differentiation between mutant and mother variety of rice by RAPD was reported by Hakim (1996). Relationship between genetic diversity and hybrid performance in rice was studied by Xiao *et al.* (1996) using PCR based markers. Ten elite inbred lines (four *japonica* and six *indica*) were crossed to produce all possible hybrids excluding reciprocals. Yield potential and its components showed a significant positive correlation with genetic distance for both *indica* X *indica* crosses and *japonica* X *japonica* crosses.

Contrasting genetic diversity relationships in rice (*Oryza sativa* L.) using different marker types had been reported by Ford-Llyod *et al.* (1997). In this study, genetic variation between samples of *Oryza sativa* from 19 localities in Bangladesh and Bhutan were assessed using RAPDs and Inter Simple Sequence Repeat PCR (ISSR-PCR). Subtle differences were revealed in the relationships between rice groups using the marker systems. The potential use of RAPD polymorphisms in differentiating variant and phenotypically normal (*Oryza sativa* var *indica*) somaclonal progenies had been reported by Godwin *et al* (1997). Pedigree and RAPD based DNA analysis of commercial US rice cultivars had been reported by Oard *et al.* (1997). They did this in 26 elite cultivars and lines of *Oryza sativa* L. recommended for commercial production in Louisiana and elsewhere in USA. Genetic distances obtained from RAPD data were correlated with estimated kinship coefficients. The mean genetic distances based on pedigree were significantly greater than those based on RAPD data for both grain types. The use of RAPD markers for the identification of *Oryza* species within a germplasm collection had been suggested by Martin *et al.*

(1997). Ninety three accessions of *Oryza* sp. have been grouped under different species based on RAPD data.

RAPD profiles were generated using mitochondrial DNA isolated from 2 cytoplasmically male sterile lines, two restorer lines and four maintainer lines of rice (Sane *et al.*, 1997). The different lines of rice, including line IR58025A and IR62829A which contained the same wild abortive cytoplasm, were distinguishable on the basis of RAPD profile. These latter two lines were not distinguishable from each other by mitochondrial DNA RFLP analyses with as many as 16 mitochondrial DNA probes. The data illustrated the utility of RAPD technique as a powerful tool for distinguishing difference between cytoplasms of various cultivars that by other techniques appear to be similar.

RAPD analysis had been carried out for wild rice genomes (Qiang *et al.*, 1998). Fourteen DNA samples, two from cultivated *Oryza sativa* accessions and 12 from wild *Oryza* accessions, were amplified for RAPD analysis with 18 random primers. A total of 147 polymorphic bands was obtained. The genetic coefficient was calculated by UPGMA cluster analysis. It was found that *Oryza rufipogon* is closely related to *japonica* rice.

A study using RAPD markers was conducted to estimate the level of genetic diversity of four South American wild rice populations collected from Amazon forest and Western Brazil river (Boso *et al.*, 1998). Genetic variation observed within the population by RAPD was in accordance with variation calculated on the basis of F-statistics.

Genetic diversity in 18 Hansraj accessions collected from farmers fields was determined by using RAPD analysis with 10 arbitrary decamer nucleotide primers (Raghunathachari, 1998). A total of 144 bands was produced of which 95 per cent were polymorphic. A dendrogram of the RAPD data showed that genetic similarity between accessions ranged from 25 – 77.5 per cent.

Genetic variability among 26 accessions of *Porteresia coarctata,* a salt tolerant wild rice, was estimated by morphological, isozyme and RAPD marker

analyses (Pisupati, 1999). Variations in six morphological traits and six isozymes were noticed. Thirty four isozyme loci encoding 17 enzymes systems were surveyed in 10 elite cultivars and five hybrids of rice (Xuan Jun *et al.*, 1999). A high level of alloenzyme diversity was found in the samples. A total of 43 alleles were present at the loci, with a mean number of alleles per locus of 1.26.

2.2.2.2.4 Amplified Fragment Length Polymorphism(AFLP)

AFLP technology is a technique of recent origin having a potential impact on genome fingerprinting and mapping. It combines both the classical restriction based and recent PCR based approaches. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA.

AFLP markers had been successfully used to assess genetic diversity by distinguishing between the major ecotypes of barley (Schut and Stam, 1997). The usefulness of AFLP markers for cultivar analysis was also revealed by this study. The use of AFLP markers for the study of rice biodiversity had been reported by Zhu *et al.* (1998). AFLP was used as a DNA fingerprinting technique to analyse 57 rice accessions of diverse origin. About 179 polymorphic AFLP markers generated from 4 primer combinations were obtained. High efficiency and random coverage of AFLP markers were established with only five combinations of primers and RFLP anchors, a frame work linkage map was constructed.

2.2.2.2.5. Variable Number of Tandom Repeats (VNTR) (Minisatellites)

VNTR arrays are highly polymorphic but are less common than microsatellites and have larger sequence motifs extending over more than 1 kbp, making them less amenable to PCR analysis.

Zhou and Gustafson (1995a) performed DNA fingerprinting to detect genetic variation with a rice minisatellite probe in rice. Later they demonstrated the potential application of rice minisatellites in DNA fingerprinting (Zhou and Gustafson 1995b).

A study was conducted to compare different PCR-based marker systems, RAPD, VNTR and AFLP for the analysis of breeding populations generated from two diverse *Musa* breeding schemes (Crouch *et al.*, 1999). All three assays detected a high level of polymorphism between parental genotypes and within progeny population. AFLP analysis had the highest multiplex ratio while VNTR analysis detected the highest level of polymorphisms between parental genotypes and within progeny populations.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The experiments for the present investigation were conducted in the laboratories of the Centre for Plant Molecular Biology, Tamil Nadu Agricultural University during 1998-2000. The details on materials involved and the methods adopted are presented below.

3.1. Materials

A total of 22 genotypes including 14 DH lines, (six from TNRH16 and four each from TNRH19 and TNRH21), three hybrids and five parents were involved in the study. The details of materials involved are given in Table 1.

3.2. Methods

3.2.1. Growing seedlings for DNA extraction

The seeds of all 22 genotypes were germinated and allowed to grow on two layers of coarse filter papers placed at the bottom of the beaker containing Hoagland's solution (Appendix I). The leaves from twenty days old seedlings of each genotype were collected and used for enzyme and DNA extraction.

3.2.2. Isozyme studies

A total of four isozymes *viz.*, esterase, SOD, PPO and peroxidase were analysed for genetic diversity analysis. The details on the isozyme analysis of the above enzymes are described below.

3.2.2.1. Enzyme Extraction

Weighed quantity of seedling sample was ground to a paste with minimum volume of 0.02M sodium phosphate buffer (pH 7.2) which is composed of 0.1M sodium dihydrogen orthophosphate and 0.1M disodium hydrogen orthophosphate.

Table 1. Genotypes included in the study

Sl. No.	Genotypes	Parentage
1.	TNRH16/3-3	DH line
2.	TNRH16/3-5	DH line
3.	TNRH16/30-6	DH line
4.	TNRH16/30-7	DH line
5.	TNRH16/33-10	DH line
6.	TNRH16/33-11	DH line
7.	TNRH16	hybrid
8.	TNRH19/1-37	DH line
9.	TNRH19/1-48	DH line
10.	TNRH19/8-55	DH line
11.	TNRH19/8-56	DH line
12.	TNRH19	hybrid
13.	TNRH21/6-58	DH line
14.	TNRH21/6-60	DH line
15.	TNRH21/8-68	DH line
16.	TNRH21/8-70	DH line
17.	TNRH21	hybrid
18.	IR58025A	female parent of all the hybrids
19.	IR58025B	maintainer line
20.	C20R	male parent of TNRH16
21.	CR1009	male parent of TNRH19
22.	IR21567-22-19-3R	male parent of TNRH21

The homogenate was transferred to eppendorf tubes, and centrifuged at 12,500 rpm for 30 min in a refrigerated centrifuge. The supernatant was collected as enzyme extract and used for isozyme analysis by polyacrylamide gel electrophoresis.

3.2.2.2 Polyacrylamide Gel Electrophoresis (PAGE)

A non-denaturing gel (native gel, without SDS) was run to separate the isozymes. Acrylamide was polymerized with bisacrylamide as a cross linking agent in the presence of ammonium per sulphate as catalyst and a chain initiator TEMED. The electrophoresis was performed in a vertical gel slab system (BIOTECH) following the protocols described by Sadasivam and Manickam (1996).

i) Reagents used for electrophoresis

a. Monomer solution (stock 30% acrylamide solution)

Acrylamide	:	29.2g
Bisacrylamide	:	0.8g
Distilled water	:	100 ml

Stored in amber coloured bottles.

b. Separating gel buffer

Tris-HCl (1.5M)	:	45.43g
Distilled water	:	250ml

c. 8x Stacking gel buffer pH 6.8

Tris-HCl (0.5M)	:	15.1 g
Distilled water	:	250ml

d. Water saturated butanol

e. Polymerzing agents

Ammonium per sulphate (APS) 10 per cent : 0.1g/ml (freshly prepared)

TEMED (Sigma): Fresh from refrigerator.

f. Electrode buffer (Tank buffer)

Tris	:	6.0g
Glycine	:	14.4.g
Distilled water	:	1 litre

g. Sample loading buffer

Tris HCl buffer (pH 6.8)	:	5.0 ml
Sucrose	:	5.0g
Bromophenol blue (0.5%)	:	0.05 g
Water	:	5 .0 ml

Autoclaved and kept in refrigerator at 4°C.

ii) Procedure

The glass plates, comb and spacers were cleaned thoroughly with detergent and wiped with 70 per cent alcohol. The glass plates were assembled with the spacers placed on the edges in between the plates, clamped tightly with clips and sealed with cellotape.

Molten agar (1.0%) was poured along the sides of the plate and allowed to solidify so as to facilitate sealing of the edges and bottom. Separating gel (8%) mixture was prepared as follows.

Distilled water	:	9.7 ml
Acrylamide mix	:	5.3ml
Tris HCl (pH 8.8)	:	5.1 ml
APS	:	0.2 ml
TEMED	:	0.01 ml

The contents were gently mixed without forming bubbles and carefully poured in between the glass plates. A layer of water saturated butanol was added above the gel to prevent oxidation and allowed to polymerize.

Stacking gel mixture was prepared as follows (4%)

Distilled water	:	6.9 ml
Acrylamide mix	:	1.7 ml
Tris HCl (pH 6.8)	:	1.25ml
APS	:	0.1 ml
TEMED	:	0.01 ml

The contents were mixed gently and poured in between the glass plates above the separating gel. The comb was placed between the plates and allowed for polymerization. After polymerization, the comb was removed carefully and wells were washed with distilled water. Then clips were removed carefully and the glass plates with the polymerized gel was placed in the electrophoretic apparatus (BIOTECH) vertical slab gel system. The electrode buffer was added to upper and lower tanks. Pre-running of the gel was done for ½-1 h in a refrigerator to cool the gel and the buffer.

iii) Loading the sample

- Enzyme extracts (70 µl each) were mixed with the sample loading buffer (10µl) and loaded in to the wells.
- Electrophoresis was carried out at constant voltage (100V) at (4°C) in a refrigerator till the loaded samples traversed the stacking gel and after reaching the separating gel, the voltage was increased to 150V.
- The run was stopped when the dye front reached the bottom of the gel plate. The gel plate was removed from the unit.
- The gel was then gently removed from the plate without any damage and stained accordingly.

3.2.2.3. Staining Procedure

1. Esterase

After the electrophoresis, the gel was incubated in the staining solution prepared as described by Smith *et al.* (1970).

Sodium dihydrogen phosphate	:	1.4g
Disodium hydrogen phosphate	:	0.55g
Fast Blue RR salt	:	0.1g
Alpha naphthyl acetate	:	0.06g (in one ml acetone) .
Distilled water	:	100 ml

The gel was incubated in the above solution for 10-20 min at 37 °C in darkness. After the brown coloured bands had clearly appeared, the enzyme reaction was stopped by soaking the gel in a mixture of methanol, water, acetic acid and ethanol in the ratio of 10:10:2:1.

2. Super oxide dismutase (SOD)

Negative staining was followed as described by Burke and Oliver (1992). The gel was incubated for one h. in the staining solution (for active gel) in darkness. The staining solution consisted of 100 ml of 50mM Phosphate buffer (pH 7.8) with 0.1mM EDTA, 3.0mM Methionine, 0.25mM NBT (Nitroblue tetrazolium), TEMED 0.2 per cent and 30mM riboflavin. The gel was rinsed with distilled water after incubation, and then placed in distilled water and exposed to four fluorescent bulbs (total light 40 W) at a distance of 30-40 cm for five to ten minutes at 27 ± 1°C. The gel was stained bluish black while the bands appeared transparent. These bands indicated the areas of activity of the enzyme.

3. Polyphenol oxidase

Reagents

1. Phosphate buffer (pH 7.0)
2. p- Phenylenediamine
3. Acetone
4. Catechol – 0.01 M

110 mg of p-Phenylenediamine was dissolved in 3 ml phosphate buffer and 1ml acetone. This was then added to 97ml of phosphate buffer and the gel was transferred to this solution and kept in darkness in a shaker for 30 min. Then 110mg of catechol was added to this and within 10 min, brown bands appeared.(Jayaraman *et al.*, 1987).

4. Peroxidase

Reagents

1. 0.1 N acetate buffer pH 4.5
2. 110 mg of Benzidine dissolved in 2 ml acetic acid
3. $H_2 O_2$
4. 7 per cent Acetic acid

100 ml of chilled acetate buffer was taken and benzidine solution was added to it in darkness. The gel was transferred to the solution and incubated under darkness in a shaker for 10-15 min. $H_2 O_2$ was added to this solution under vigorous shaking. Blue bands appeared. The reaction was stopped by adding 7 per cent acetic acid. (Smith *et al.*, 1988).

3.2.3 Random Amplified Polymorphic DNA (RAPD) analysis

3.2.3.1 Isolation of genomic DNA

The genomic DNA was isolated from two grams of fresh leaf samples collected from each genotype following the procedure by Melody (1997).

Reagents and solutions

i) DNA extraction buffer (100ml)

CTAB (Cetyl Trimethyl Ammonium Bromide)	:	12% w/v
Tris HCl (pH8.0)	:	100mM
Sodium chloride	:	1.4mM
EDTA	:	20mM
2-mercaptoethanol	:	0.1% v/v
(added immediately prior to use)		

ii) Stock solutions

1) 1M Tris HCl (pH 8.0)

Tris base (12.11g) was dissolved in 50 ml of distilled water and pH was adjusted to 8.0 with concentrated HCl. The solution was cooled to room temperature

before making final adjustment of the pH. The volume of the solution was adjusted to 100 ml with sterile distilled water.

2) 3M Sodium acetate (pH 5.2)

Sodium acetate (40.81g) was dissolved in 60 ml of sterile distilled water. The pH was adjusted to 5.2 with glacial acetic acid and final volume was made upto 100 ml with sterile distilled water.

3) 0.5 M EDTA (pH 8.0)

Disodium ethylene diamine triacetate (18.61g) was added to 50 ml of distilled water. It was then stirred vigorously with a magnetic stirrer with addition of NaOH pellets and pH was adjusted to 8.0. The final volume was made to 100ml with sterile distilled water.

4) TE buffer

Tris-HCl (pH 8.0) : 10mM

EDTA (pH 8.0) : 1mM

This was dissolved and made upto 100ml, autoclaved and stored at 4 °C.

5)Ice-cold isopropanol

Isopropanol stored in fridge was used.

6) Chloroform-isoamyl alcohol (24:1 v/v)

To 24 parts of chloroform, one part of isoamyl alcohol was added and mixed properly.

7) Ethanol 100 per cent and 70 per cent

Absolute ethanol was used as such for 100 per cent ethanol. 70 per cent ethanol was prepared by adding 30 parts of double distilled water to 70 parts of absolute ethanol.

8) RNase A

10 mg/ml RNase was dissolved in TE or sterile water and was boiled for 15 min at 100 °C to destroy DNase and stored at –20 °C.

iii) Protocol for extraction of genomic DNA

1. Prior to extraction, the pestle and mortar, spatula and scissors were steam sterilized.

2. Leaf samples (2g) were cut into bits with the help of sterile scissors and transferred to prechilled pestle and mortar.
3. The leaf tissue was frozen with the addition of liquid nitrogen and ground to a fine powder using the pestle.
4. The fine powder was allowed to thaw in presence of 5-10ml of pre-heated extraction buffer in poly propylene centrifuge tubes and incubated for 30 min at 65 °C with occasional mixing in a water bath.
5. The tubes were removed from the water bath and equal volume of chloroform-isoamyl alcohol mixture (24:1 v/v) was added and mixed by gentle inversion for 15 min.
6. It was centrifuged at 10,000 rpm for 20 min at room temperature.
7. The clear phase was transferred to a new tube.
8. Equal volume of ice-cold isopropanol was added and mixed gently by inversion until DNA was precipitated out (10-20 seconds).
9. The precipitated DNA was hooked out using a bent pasteur pipette, rinsed in 70 per cent ethanol and air dried.
10. The DNA pellet was dissolved in 250-500 µl of TE and RNase was added (10 µl) and incubated at 37 °C for ½- 1 h.
11. Then equal volume of chloroform-isoamylalcohol was added, mixed thoroughly and certrifuged at 12,500 rpm for 10 min.
12. The upper aqueous layer was taken, twice the volume of absolute ethanol and 25 µl of sodium acetate was added and kept overnight for DNA precipitation.
13. Then the tubes were spinned at 12,500 rpm for 5-10 min, and supernatant was discarded.
14. The pellet was washed with 70 per cent ethanol for 20 seconds and air dried.
15. The fresh pellet was dissolved in 100 µl of TE and stored at – 20 °C.

3.2.3.2. Quantification of DNA

The DNA extracted from all the genotypes were quantified by adopting the protocol of Brunk *et al.*(1979).

a) Reagents

1. 10x TNE buffer

Tris	:	100mM
EDTA	:	10mM
Sodium chloride	:	2mM

These three chemicals were dissolved in 60 ml of sterile distilled water and pH was adjusted to 7.4 and the final volume was made upto 100 ml using sterile distilled water, autoclaved and stored at 4 °C.

2. Hoechst 33258 dye
3. Calf thymus Standard DNA
4. Fluorometer (Model: Hoefer DyNA quant 200)

b) Protocol for quantification of DNA

1. 100 ml of 1x TNE buffer was prepared and to this 10 µl dye was added and mixed thoroughly.
2. 2 ml of this buffer was taken in a clear quartz cuvette and calibrated to read 'zero'(blank).
3. 2 µl of calf thymus standard DNA was added to the blank and calibrated to 100 ng per µl .
4. The cuvette was washed with 1 ml 1x TNE buffer.
5. 2 µl of unknown DNA sample was added to 2 ml of 1x TNE buffer.
6. The quantity of DNA present in the sample was read as 'x' mg/µl at 260 nm.

3.2.3.3. PCR amplification using random primers

A total of 30 random decamer primers (Table 2) were used to amplify specific regions in the 22 genotypes using the following conditions.

Table 2. Particulars of sequences of operon primers used

Sl.No.	Primer	Sequence
1.	OPAH01	5'TCCGCAACCA3'
2.	OPAH11	5'TCCGCTGAGA3'
3.	OPAH18	5'GGGCTAGTCA3'
4.	OPAH20	5'GGAAGGTGAG3'
5.	OPC02	5'GTGAGGCGTC3'
6.	OPC03	5'GGGGGTCTTT3'
7.	OPC07	5'GTCCCGACGA3'
8.	OPC09	5'CTCACCGTCC3'
9.	OPC10	5'TGTCTGGGTG3'
10.	OPC13	5'AAGCCTCGTC3'
11.	OPC14	5'TGCGTGCTTG3'
12.	OPC16	5'CACACTCCAG3'
13.	OPC18	5'TGAGTGGGTG3'
14.	OPF06	5'GGGAATTCGG3'
15.	OPF07	5'CCGATATCCC3'
16.	OPF08	5'GGGATATCGG3'
17.	OPF10	5'GGAAGCTTGG3'
18.	OPF14	5'TGCTGCAGGT3'
19.	OPF15	5'CCAGTACTCC3'
20.	OPF19	5'CCTCTAGACC3'
21.	OPP02	5'TCGGCACGCA3'
22.	OPP05	5'CCCCGGTAAC3'
23.	OPP11	5'AACGCGTCGG3'
24.	OPU05	5'TTGGCGGCCT3'
25.	OPU06	5'ACCTTTGCGG3'
26.	OPU07	5'CCTGCTCATC3'
27.	OPU10	5'ACCTCGGCAC3'
28.	OPU15	5'ACGGGCCAGT3'
29.	OPU16	5'CTGCGCTGGA3'
30.	OPZ14	5'TCGGAGGTTC3'

Reaction mixture (15 µl) contained

DNA (25 ng/ µl)	:	2 .0 µl
dNTPs (2.5mM)	:	1.2 µl
Primer	:	1.44 µl
$MgCl_2$	:	0.18 µl
10x assay buffer	:	1.5 µl
Taq Polymerase	:	0.18 µl
Sterile distilled water	:	8.5 µl

The reaction mixture was given a momentary spin for thorough mixing of the cocktail components. Then the PCR tubes were loaded in a thermal cycler.

The thermal cycler program followed for DNA amplification is given below.

Profile 1 : 94° C for 5 min - initial denaturation
Profile 2 : 94 °C for 1 min - denaturing
Profile 3 : 37 °C for 1 min - annealing — 35 cycles
Profile 4: 72 °C for 1 min - extension
Profile 5: 72 °C for 7 min - final extension
Profile 6 : 4 °C for infinity to hold the samples.

3.2.3.4. Agarose gel electrophoresis

Agarose gel electrophoresis (Sambrook *et al.*, 1989) was performed to check the quality of DNA and also to separate the amplified products.

Materials

a. Agarose : 0.8 per cent (for genomic DNA)
b. Agarose : 1.5 per cent (for PCR samples)
c. 5x TBE buffer (pH 8.0)

Tris HCl	:	54.0 g
Boric acid	:	27.5g
0.5 M EDTA (pH 8.0)	:	200 ml
Distilled water	:	1000 ml

The solution was prepared, autoclaved and stored at room temperature.

d. Electrophoresis unit, powerpack, casting tray, comb.

e. Loading/Tracking dye

Glycerol - 3 ml

Water - 7 ml

Bromophenol blue - 0.025 g

Autoclaved and kept in fridge at 4 °C

f. Staining solution

g. uv transilluminator

h. Alpha imager TM 1200 documentation and analysis system.

Protocol

1. One and a half litre of electrophoresis buffer (1x TBE) was prepared to fill the electrophoresis tank and to prepare the gel.
2. The open ends of the pyrex gel casting plate were sealed with a cellotape and placed on a perfectly horizontal leveled platform.
3. Agarose (0.8% for genomic DNA and 1.5% for PCR) was added to 1x TBE, boiled till the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was also added as an intercalating agent of DNA which will help in its visualization in uv rays.
4. It was then poured into the gel mould and the comb was placed properly and allowed to solidify.
5. The casted gel was immersed in the buffer tank with the wells towards the cathode and submerged with 1x TBE to a depth of 1 cm.
6. The PCR amplified product was mixed with the loading buffer and loaded in the wells. Lambda DNA (EcoRI and Hind III double digest) was also added in one of the wells as a standard marker.
7. The cathode and anode were connected to powerpack and the gel was run at a constant voltage of $5V/cm^2$.
8. The gel was allowed to run until the tracking dye reached at about 2 cm from the anode end.

3.2.3.5. Gel Photodocumentation

PCR amplification products were separated by electrophoresis in 1.5 per cent agarose gels, stained with ethidium bromide and viewed and photographed using Alpha Imager TM 1200 documentation and analysis system.

3.2.4. Data analysis

For each accession, an isozyme band presence/absence profile was recorded in all the four isozyme systems. The score of one and zero were given for the presence and absence of the band respectively. The same method was followed for the bands generated with 30 random primers across the 22 genotypes. The cluster analysis was carried out using NTSYSpc version 1.7 (Rohlf, 1992).

EXPERIMENTAL RESULTS

CHAPTER IV

EXPERIMENTAL RESULTS

The results obtained from the genetic diversity analysis of the 22 genotypes involving four isozymes and 30 random primers are presented below.

4.1. Isozyme analysis

4.1.1. Esterase

A total of three isoforms were resolved in each of the 22 genotypes involved. These isoforms were monomorphic among all the 22 genotypes. No difference in banding pattern was noticed across the DH lines, hybrids and their respective parents. The isozyme banding pattern is shown in Plate 1.

4.1.2. Super oxide dismutase

A total of five isoforms were observed and they were present in all the lines uniformly indicating monomorphism between DH lines, hybrids and the parents. Apart from these five specific bands, some non-specific bands were also present in some lines in the top of the gel. The isozyme banding pattern is shown in Plate 2.

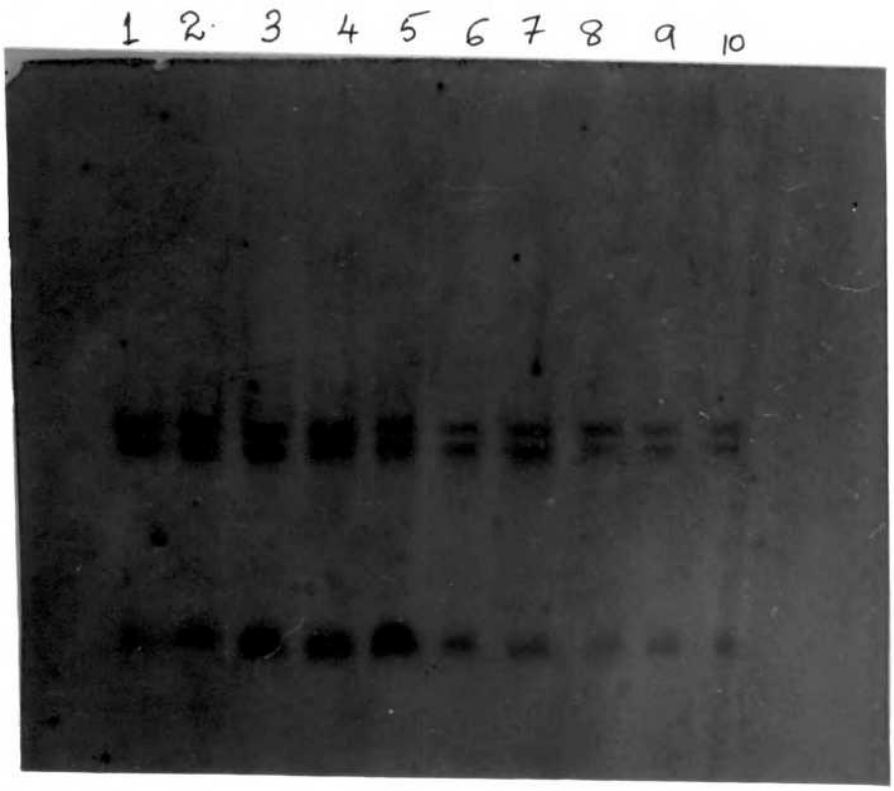
4.1.3. Polyphenol oxidase

A maximum of six isoforms were observed for this enzyme. Differences were present in the lower three isoforms (Fig. 1). The upper three isoforms were present uniformly in all the lines. The sixth isoform was a low molecular weight one which is having a relative mobility of 100 per cent. The absence of lower three isoforms in some of the DH lines *viz.*, TNRH16/30-6, TNRH16/30-7, TNRH19/8-55, TNRH21/6-60, TNRH21/8-70 and hybrid TNRH21 is misleading, since all the parents involved in the study had the bands. The isozyme banding pattern is shown in Plate 3.

4.1.4. Peroxidase

A total of nine isoforms of peroxidase was observed. Only in a few lines like TNRH19/1-37, TNRH19/8-55 and IR58025B the lower isoform Prx9 was absent (Fig.2). The differences in banding pattern of peroxidase was observed with regard to low molecular weight bands. However the female parent of TNRH 19, IR58025A had

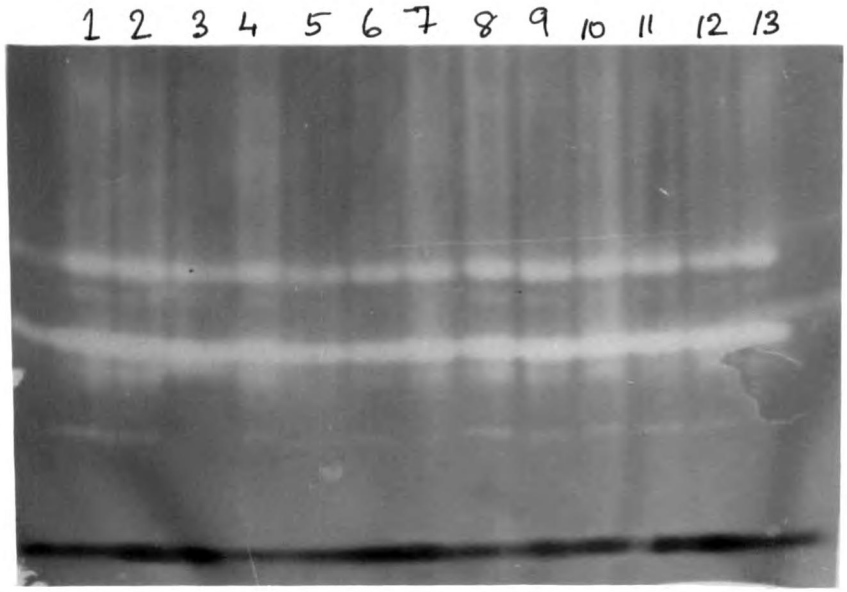
Plate 1. Banding pattern for esterase isozyme



LEGEND

1-	IR58025A	6-	TNRH16/3-5
2-	IR58025B	7-	TNRH16/30-6
3-	C20R	8-	TNRH16/30-7
4-	TNRH16	9-	TNRH16/33-10
5-	TNRH16/3-3	10-	TNRH16/33-11

Plate 2. Banding pattern for SOD isozyme



LEGEND			
1-	IR58025A	7-	TNRH19/8-56
2-	CR1009	8-	IR21567R
3-	TNRH19	9-	TNRH21
4-	TNRH19/1-37	10-	TNRH21/6-58
5-	TNRH19/1-48	11-	TNRH21/6-60
6-	TNRH19/8-55	12-	TNRH21/8-68
		13-	TNRH19/8-70

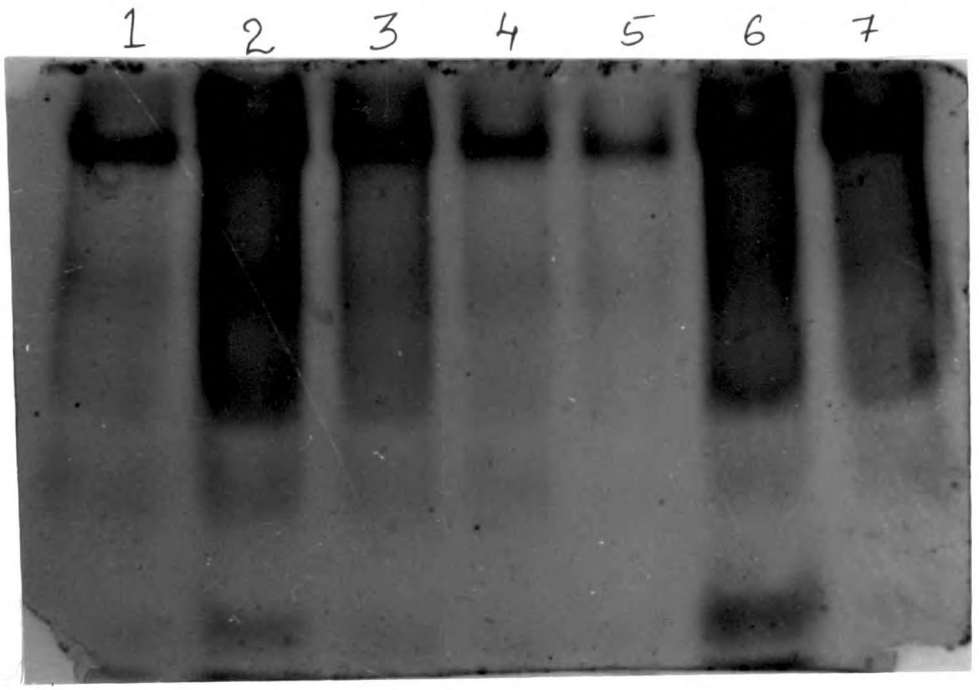
Fig. 1. Zymogram pattern for PPO across 22 genotypes

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	0	—	—	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—
—	—	—	0	0	—	—	—	—	—	0	—	0	—	0	—	0	—	—	—	—	—
—	—	—	0	0	—	—	—	—	—	0	—	0	—	0	—	0	—	—	—	—	—

LEGEND

1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

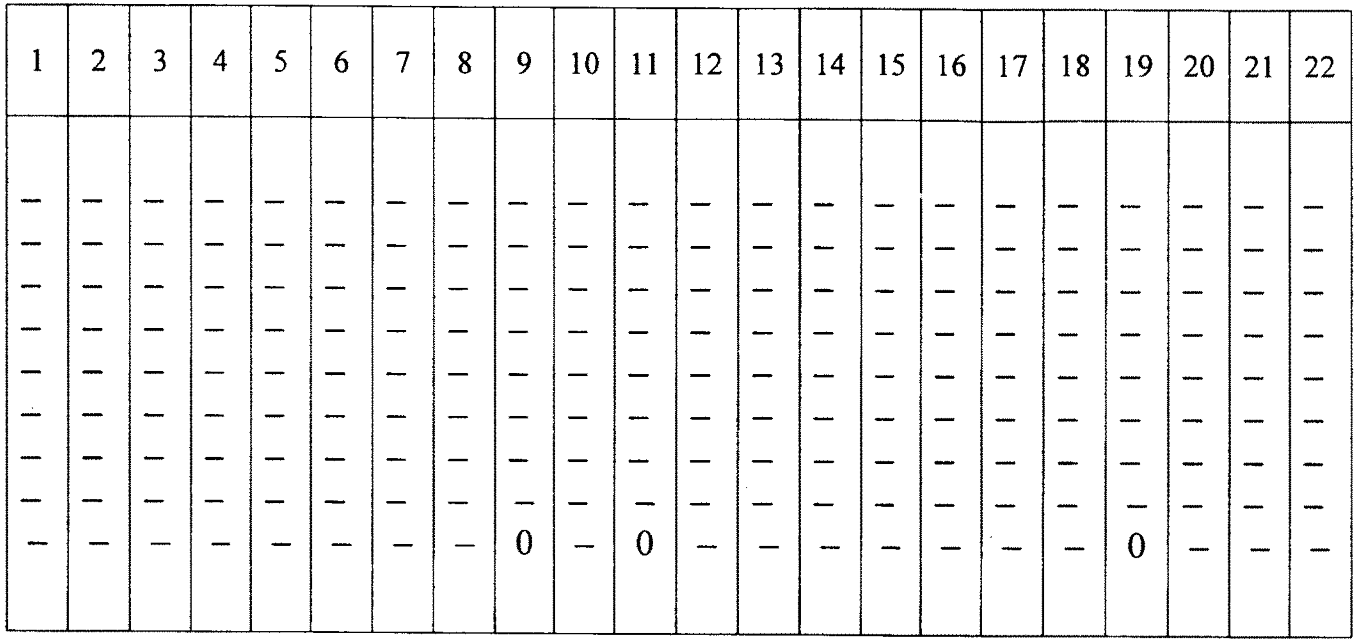
Plate 3. Banding pattern for PPO isozyme



LEGEND

1-	TNRH16	5-	TNRH16/30-7
2-	C20R	6-	TNRH16/33-10
3-	TNRH16/3-5	7-	TNRH16/33-11
4-	TNRH16/30-6		

Fig. 2. Zymogram pattern for peroxidase across 22 genotypes



LEGEND

1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

the band. Hence, its absence in the hybrid is misleading as isozyme is a codominant marker. The isozyme banding pattern is shown in Plate 4.

The number of isozyme markers and their level of polymophism observed are given in Table 3.

4.2 RAPD Analysis.

The RAPD analysis of all the 22 genotypes indicated that the level of polymorphism was comparitively higher with RAPD markers. The 30 random primers produced a total of 280 markers, of which 87 were polymorphic. The average number of markers produced per primer was nine. The maximum number of RAPD markers were generated by the primer OPF10 (13 markers) and the least number by OPF14 and OPAH 20 (3-5 markers). The different levels of polymorphism across the 22 genotypes exhibited by 30 random primers are presented in the Table 4.

4.2.1. RAPD profile observed with selected primers

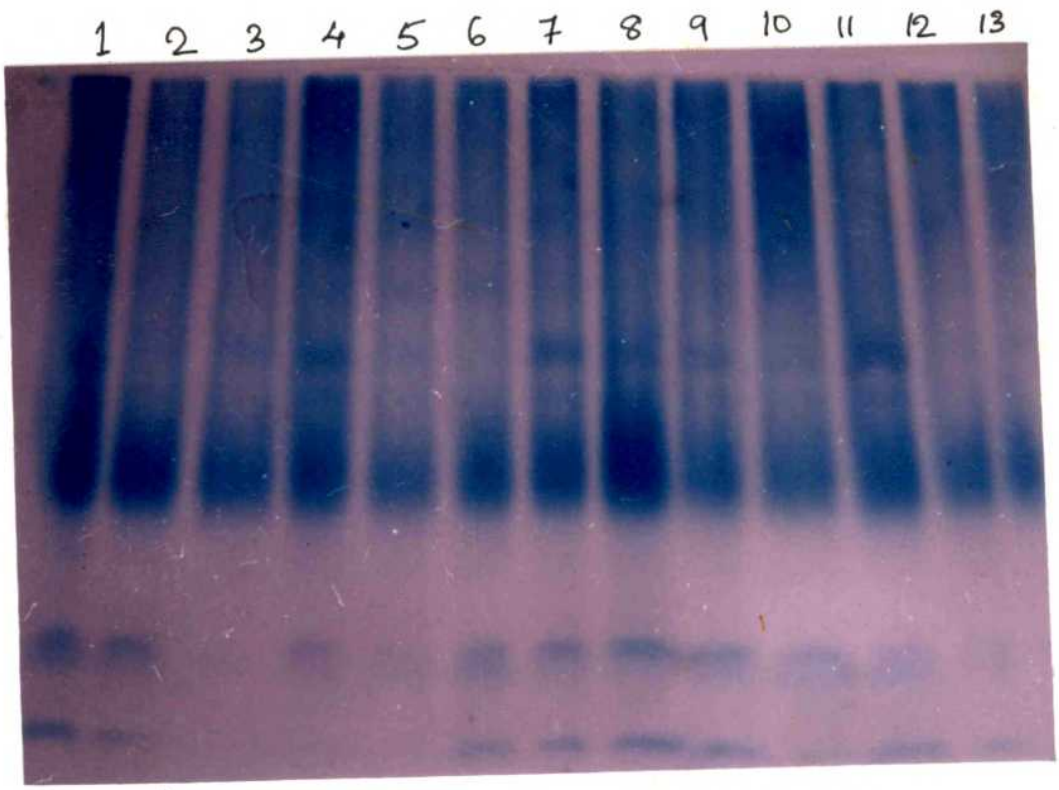
1. Primer OPAH18

The number of bands produced varied from 7 in most of the lanes to 10 (in TNRH16/33-6 and TNRH16/33-7). A total of 10 markers were produced and of these three were polymorphic. The percentage of polymorphism observed was 30 per cent. OPAH18-5 and OPAH18-6 markers were present only in TNRH16 and its derivatives and hence these bands can be used to identify TNRH16 and its derivatives. (Plate 5).

2. Primer OPF10

This was a primer which showed some distinct variation among the DH lines. OPF10-5 marker is present in all the lines except derivatives of TNRH16 – TNRH16/3-3, TNRH16/3-5, TNRH16/30-6, TNRH16/30-7, TNRH16/33-10 and TNRH16/33-11. Absence of this fifth marker is noticed in its restorer line C20R also. As C20R is the male parent of TNRH16, it may be inherited from this parent. Similarly, OPF10-11 marker is absent in all the above said lines while it is present uniformly in all other lines. Another peculiarity is that, OPF10-4 marker is present in

Plate 4. Banding pattern for peroxidase isozyme



LEGEND			
1-	IR58025A	7-	TNRH19/8-56
2-	CR1009	8-	IR21567R
3-	TNRH19	9-	TNRH21
4-	TNRH19/1-37	10-	TNRH21/6-58
5-	TNRH19/1-48	11-	TNRH21/6-60
6-	TNRH19/8-55	12-	TNRH21/8-68
		13-	TNRH19/8-70

Table 3 Total number of isozyme markers observed and their level of polymorphism

Sl.No.	Enzyme	Monomorphic	Polymorphic	Total	Percentage of polymorphism
1	Esterase	3	0	3	0.0
2	SOD	5	0	5	0.0
3	Polyphenoloxidase	3	3	6	50.0
4	Peroxidase	8	1	9	11.1
	Total	19	4	23	17.39

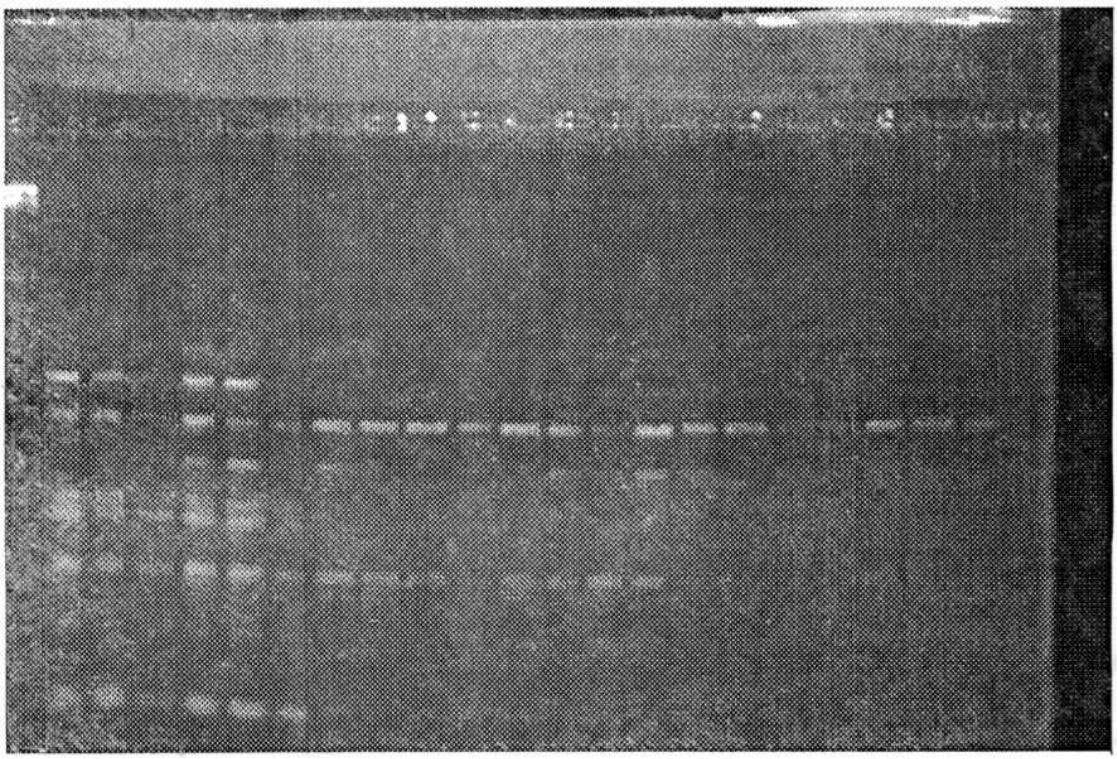
Table 4. Total number of RAPD markers observed and their level of polymorphism

Sl.No.	Primer	Number of monomorphic markers	Number of polymorphic markers	Total number of markers	Percentage of polymorphism
1.	OPAH01	6	2	8	25.0
2.	OPAH11	6	4	10	40.0
3.	OPAHI8	7	3	10	30.0
4.	OPAH20	3	2	5	40.0
5.	OPC02	12	0	12	0.0
6.	OPC03	12	0	12	0.0
7.	OPC07	5	3	8	37.5
8.	OPC09	6	1	7	14.3
9.	OPC10	5	3	8	37.5
10.	OPC13	7	2	9	22.2
11.	OPC14	7	2	9	22.2
12.	OPC16	4	3	7	42.8
13.	OPC18	7	1	8	12.5
14.	OPF06	4	6	10	60.0
15.	OPF07	5	7	12	58.3
16.	OPF08	7	3	10	30.0
17.	OPF10	7	6	13	46.2
18.	OPF14	3	2	5	40.0
19.	OPF15	11	2	13	15.4
20.	OPF19	2	2	4	50.0
21.	OPP02	8	3	11	27.3
22.	OPP05	6	0	6	0.0
23.	OPP11	6	0	6	0.0
24.	OPU05	10	4	14	28.6
25.	OPU06	4	5	9	55.6
26.	OPU07	6	2	8	25.0
27.	OPU10	6	5	11	45.5
28.	OPU15	6	3	9	33.3
29.	OPU16	7	3	10	30.0
30.	OPZ14	7	9	16	56.3
	Total	193	87	280	31.07

Plate : 5

RAPD profile for the primer OPAH13

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22



LEGEND

M- lamba double digest
1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

only TNRH16 and its derivatives. So this primer can be specifically used for identification of TNRH16 derivatives. A total of 13 markers were produced by this primer and of these six were polymorphic. The percentage of polymorphism produced was 46.2 per cent (Plate 6).

3. Primer OPP02

A total number of 11 markers were produced, of which three were polymorphic. This accounted for a polymorphism of 27.3 per cent. The number of bands produced varied from 8-11. OPP02-1 marker is present only in TNRH19 and TNRH21 derivatives and it is completely absent in TNRH16 and its derivatives. This marker was also absent in C20R. This further confirms the relatedness of TNRH16 DH lines with the male parent. The rest of the banding pattern is almost similar in all the lines (Plate 7).

4. Primer OPU05

The number of bands produced varied from 11-14. A total number of 14 markers were produced. Of these, four were polymorphic, accounting for 28.6 percentage of polymorphism. OPU05-9 marker was present only in TNRH16/3-5 and TNRH21. The number of bands produced was more showing the more number of positions in the genome with homology to the primer sequence (Plate 8).

5. Primer OPU07

The number of bands produced ranged from 4-8. A total of eight markers were produced and of these two were found to be polymorphic and the percentage of polymorphism was 25.0 per cent. OPU07-3 marker was present only in TNRH16, TNRH16/3-5, TNRH 19, TNRH19/1-37, TNRH 19/1-48, C20R and CR1009 (Plate 9).

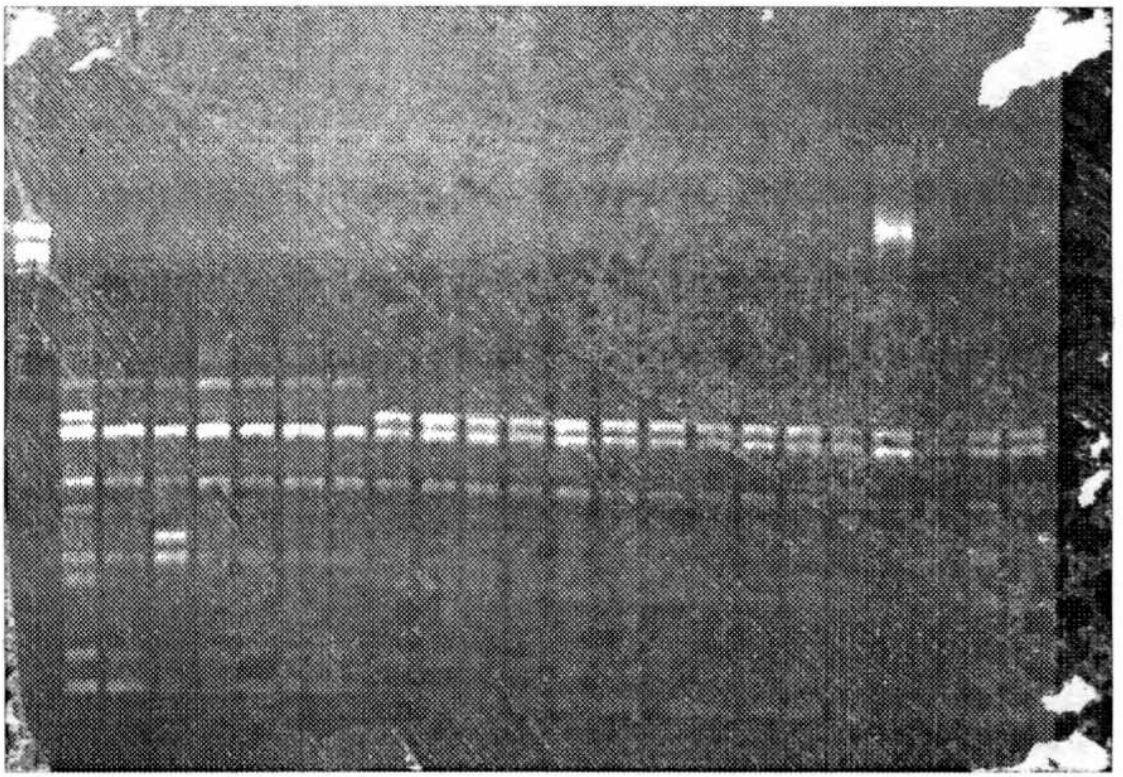
6. Primer OPZ14

The number of bands produced by this primer varied from 7-15. TNRH16 showed a peculiar banding pattern and contained around 15 bands, whereas in all other lanes, the number of bands produced were 7,8 or 9. A total of 16 markers were produced and of these nine were polymorphic accounting for 56.3 per cent

Plate : 6

RAPD profile for the primer OPF10

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22



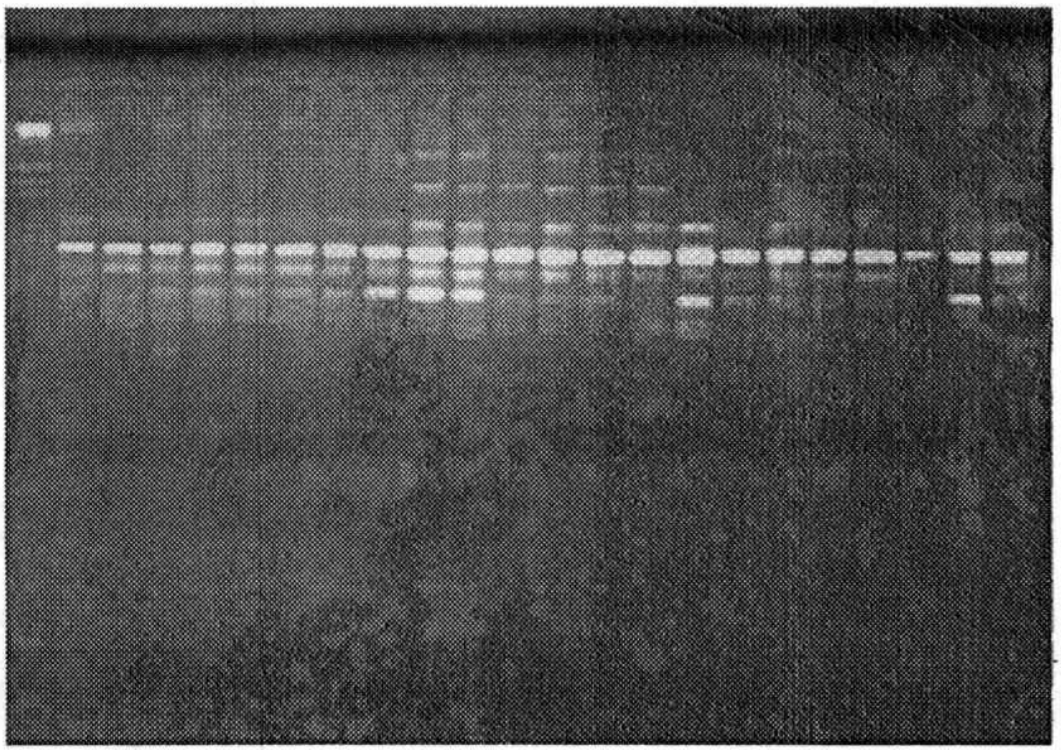
LEGEND

M- lamba double digest
1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

Plate 7

RAPD profile for the primer OPP02

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22



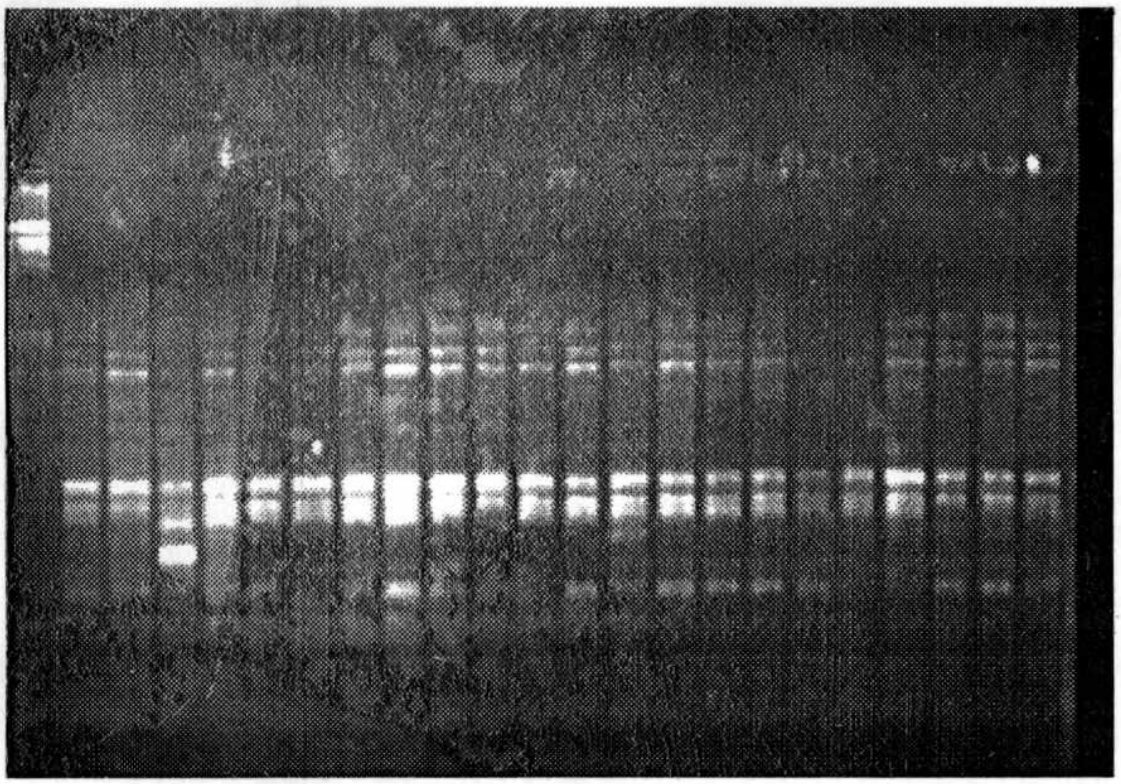
LEGEND

M- lambda double digest
1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

Plate : 8

RAPD profile for the primer OPU05

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

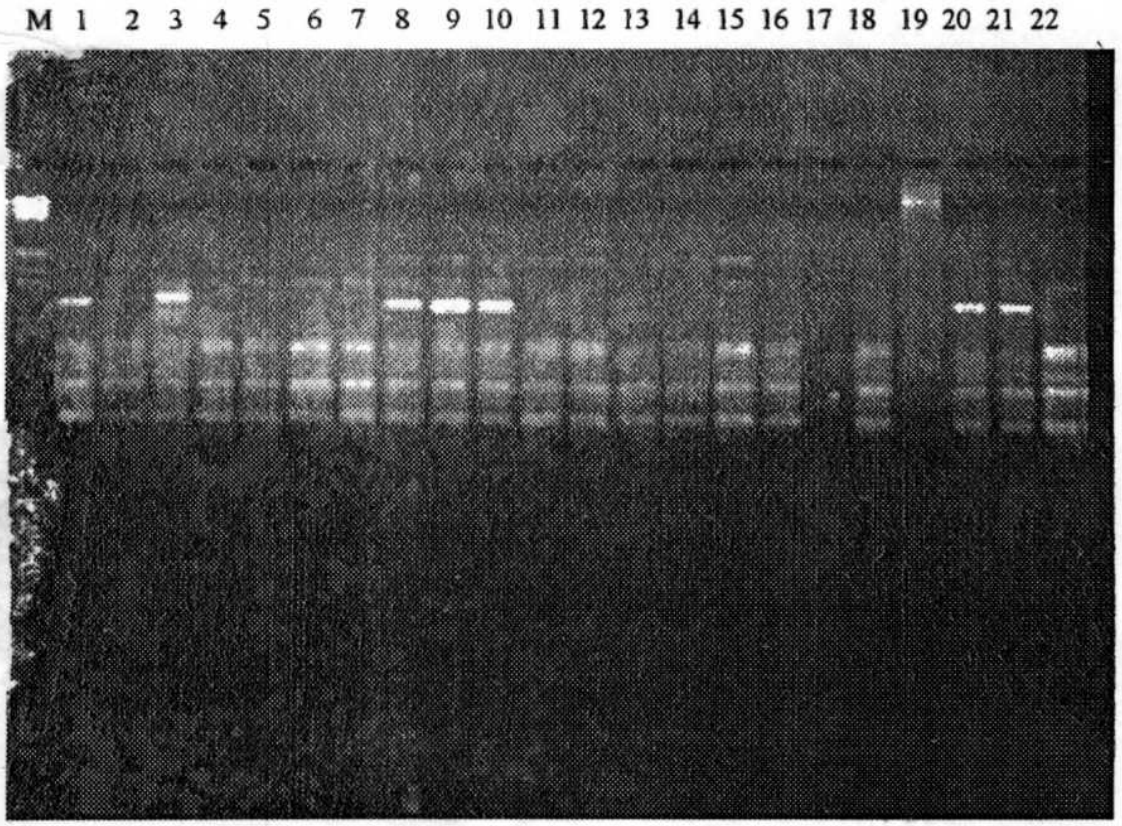


LEGEND

M- lamba double digest
1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

Plate : 9

RAPD profile for the primer OPU 07



LEGEND

M- lamba double digest
1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

polymorphism. The main factor accounting for the polymorphism is the presence of extra bands in TNRH16 (Plate 10).

4.3 Comparison between isozyme and RAPD analyses

The four isozymes analysed produced a total of 23 markers of which only four were polymorphic across the genotypes studied. The two enzyme systems *viz.*, esterase and SOD were monomorphic. The polymorphic enzyme markers found with PPO and peroxidase analysis were not clear. The absence of low molecular weight marker of peroxidase in TNRH19/1-37 and TNRH19/8-55 is not clear when both the parents and the hybrid were having the marker. Same is the condition regarding the polymorphic markers produced by PPO. Some of low molecular weight markers (2-3) are absent in DH lines but present in all the parents and hybrids involved (except TNRH21).

The RAPD analysis with 30 random primers among the 22 genotypes revealed clear cut polymorphism. A total of 280 markers were produced, of which 87 were polymorphic (31.07%). The average number of markers produced per primer was about nine and the range of markers produced was from a minimum of four (OPF14) to a maximum of 16 (OPZ14).

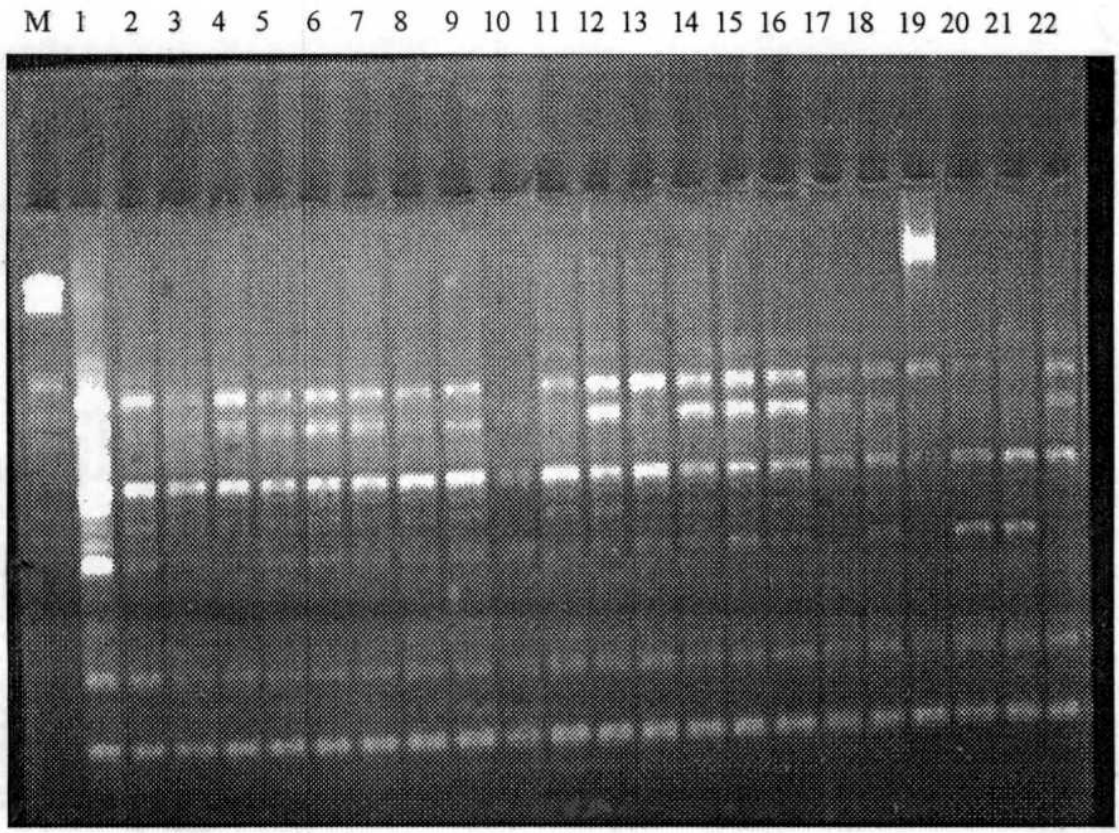
4.4 Estimation of similarity index and cluster analysis.

The estimation of similarity indices based on the isozyme marker data revealed that many of the DH lines were 100 per cent similar to one another except the lines TNRH16/30-7 with TNRH19/1-37, TNRH16/30-7 with IR58025B and TNRH21/6-60 with IR58025B. These lines possessed a similarity index value of 82.6 per cent (Table 5).

The band sharing data from RAPD analysis revealed better range of polymorphism when compared to isozyme markers. However, the individuals analysed showed 82 per cent similarity among themselves (Table 6).

Plate : 10

RAPD profile for the primer OPZ14



LEGEND

M- lamba double digest
1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

Table 5 Similarity Co-efficients between pairs of genotypes based on isozyme data

1	1.000																					
2	1.000	1.000																				
3	1.000	1.000	1.000																			
4	0.913	0.913	0.913	1.000																		
5	0.870	0.870	0.870	0.957	1.000																	
6	1.000	1.000	1.000	0.913	0.870	1.000																
7	1.000	1.000	1.000	0.913	0.870	1.000	1.000															
8	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000														
9	0.957	0.957	0.957	0.870	0.826	0.957	0.957	0.957	1.000													
10	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000												
11	0.870	0.870	0.870	0.957	0.913	0.870	0.870	0.870	0.913	0.870	1.000											
12	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000										
13	0.913	0.913	0.913	1.000	0.957	0.913	0.913	0.913	0.870	0.913	0.957	0.913	1.000									
14	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000	0.913	1.000								
15	0.870	0.870	0.870	0.957	1.000	0.870	0.870	0.870	0.826	0.870	0.913	0.870	0.957	0.870	1.000							
16	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000	0.913	1.000	0.870	1.000						
17	0.913	0.913	0.913	1.000	0.957	0.913	0.913	0.913	0.870	0.913	0.957	0.913	1.000	0.913	0.957	0.913	1.000					
18	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000	0.913	1.000	0.870	1.000	0.913	1.000				
19	0.957	0.957	0.957	0.870	0.826	0.957	0.957	0.957	1.000	0.957	0.913	0.957	0.870	0.957	0.826	0.957	0.870	0.957	1.000			
20	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000	0.913	1.000	0.870	1.000	0.913	1.000	0.957	1.000		
21	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000	0.913	1.000	0.870	1.000	0.913	1.000	0.957	1.000	1.000	
22	1.000	1.000	1.000	0.913	0.870	1.000	1.000	1.000	0.957	1.000	0.870	1.000	0.913	1.000	0.870	1.000	0.913	1.000	0.957	1.000	1.000	1.000
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

LEGEND

1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

Table 6 Similarity Co-efficients between pairs of genotypes based on RAPD data

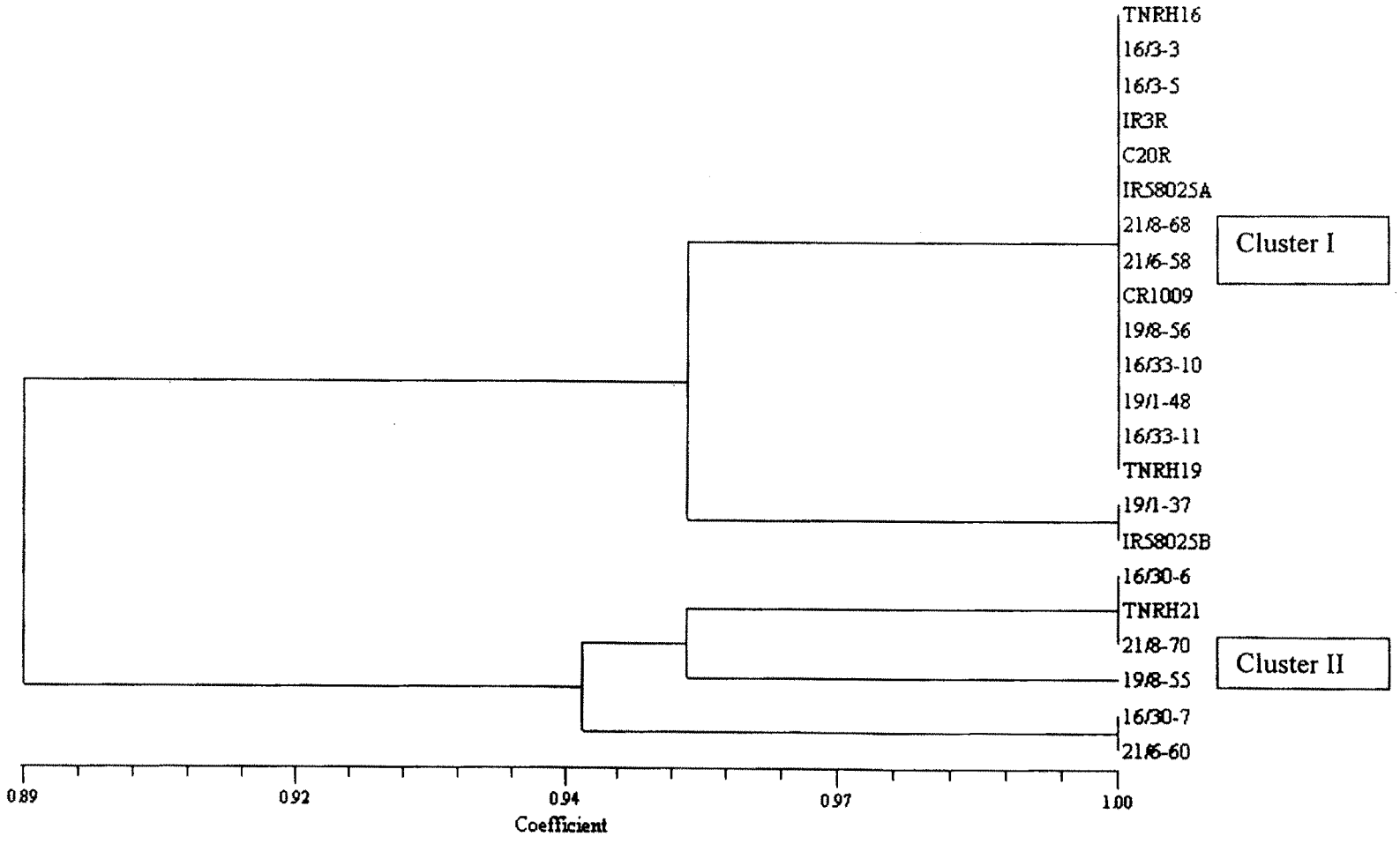
1.	1.000																						
2.	0.900	1.000																					
3.	0.875	0.896	1.000																				
4.	0.871	0.914	0.896	1.000																			
5.	0.868	0.911	0.900	0.968	1.000																		
6.	0.861	0.918	0.886	0.961	0.943	1.000																	
7.	0.861	0.925	0.879	0.939	0.929	0.943	1.000																
8.	0.846	0.846	0.850	0.882	0.871	0.857	0.907	1.000															
9.	0.857	0.843	0.846	0.886	0.861	0.861	0.889	0.954	1.000														
10.	0.861	0.839	0.843	0.882	0.857	0.871	0.886	0.943	0.954	1.000													
11.	0.861	0.854	0.843	0.889	0.879	0.886	0.886	0.921	0.946	0.950	1.000												
12.	0.861	0.861	0.836	0.896	0.893	0.893	0.907	0.907	0.911	0.907	0.929	1.000											
13.	0.861	0.854	0.836	0.882	0.893	0.886	0.900	0.914	0.896	0.893	0.914	0.943	1.000										
14.	0.850	0.857	0.846	0.907	0.911	0.911	0.918	0.911	0.900	0.896	0.918	0.968	0.961	1.000									
15.	0.850	0.850	0.846	0.907	0.889	0.904	0.904	0.911	0.893	0.896	0.911	0.954	0.925	0.950	1.000								
16.	0.864	0.871	0.861	0.914	0.889	0.911	0.918	0.904	0.893	0.904	0.911	0.946	0.918	0.943	0.964	1.000							
17.	0.836	0.857	0.832	0.886	0.882	0.896	0.904	0.911	0.893	0.896	0.911	0.946	0.932	0.943	0.957	0.957	1.000						
18.	0.854	0.861	0.843	0.882	0.864	0.900	0.893	0.893	0.889	0.893	0.907	0.921	0.907	0.911	0.932	0.946	0.939	1.000					
19.	0.825	0.846	0.836	0.868	0.864	0.879	0.886	0.900	0.875	0.864	0.893	0.914	0.893	0.918	0.939	0.918	0.925	0.950	1.000				
20.	0.818	0.846	0.829	0.861	0.857	0.864	0.893	0.893	0.868	0.857	0.871	0.900	0.886	0.896	0.918	0.904	0.932	0.921	0.936	1.000			
21.	0.832	0.832	0.843	0.846	0.836	0.857	0.879	0.900	0.889	0.886	0.886	0.893	0.879	0.889	0.911	0.904	0.925	0.914	0.907	0.943	1.000		
22.	0.821	0.829	0.832	0.857	0.854	0.875	0.882	0.896	0.879	0.875	0.896	0.904	0.904	0.914	0.914	0.921	0.929	0.946	0.939	0.932	0.932	1.000	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	

LEGEND

1- TNRH 16
2- TNRH16/3-3
3- TNRH16/3-5
4- TNRH16/30-6
5- TNRH16/30-7
6- TNRH16/33-10
7- TNRH16/33-11
8- TNRH19
9- TNRH19/1-37
10- TNRH19/1-48
11- TNRH19/8-55
12- TNRH19/8-56
13- TNRH21
14- TNRH21/6-58
15- TNRH21/6-60
16- TNRH21/8-68
17- TNRH21/8-70
18- IR58025A
19- IR58025B
20- C20R
21- CR1009
22- IR21567R

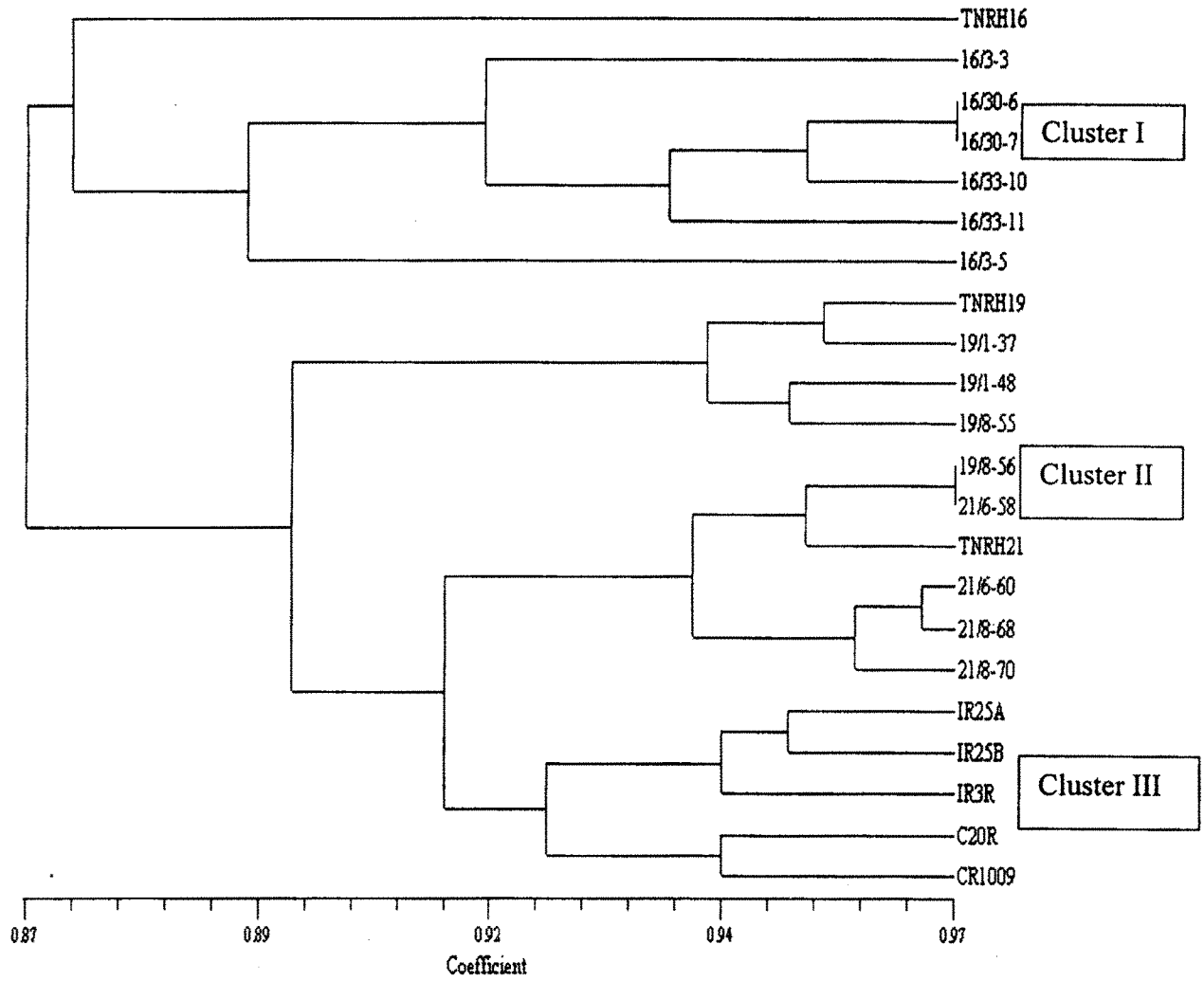
Cluster analysis was performed on similarity coefficient matrices calculated from isozyme and RAPD markers. The dendrogram based on isozymes separated the 22 genotypes into two groups. (Fig. 3) and does not show a clear cut grouping of genotypes. The dendrogram based on RAPD markers showed three different clusters (Fig. 4). The cluster number I had the TNRH16 hybrid and its six DH lines. The cluster number II possessed the hybrids TNRH19 and TNRH21 with their DH lines, whereas cluster number III had all the five parents viz., IR58025A, IR58025B, C20R, CR1009 and IR21567R.

Fig. 3. Dendrogram based on isozyme analysis



Key : IR3R : IR21567-22-19-3R

Fig.4 Dendrogram based on RAPD data



Key : IR3R : IR21567-22-19-3R

DISCUSSION

CHAPTER V

DISCUSSION

Genetic markers have been observed and used since the dawn of genetics. More recently, as genetic marker technology progressed from morphological markers to molecular markers, markers have been used to investigate wide range of problems in biology. Genetic markers are employed to determine relatedness between individuals. Much of this work has employed isozymes, largely because they are simply detected, at low cost. Genetic markers also can be used to portray diversity within a group of individuals (Smith, 1988), and to identify groupings of cultivars which are of similar kind (Souza and Sorrells, 1989) or perform similarity in crosses to other cultivars (Lee *et al.*, 1989). By understanding the potentials of genetic markers, the present investigation was conducted to understand the relatedness between DH lines of different hybrids and diversity of DH lines of each hybrid involving a set of four isozymes and 30 random primers. The results obtained in the study are discussed below.

In the present investigation, a total number of 14 anther culture derived DH lines (six from TNRH16, and four each from TNRH19 and TNRH21) were analysed for their profiles of isozymes *viz.*, esterase, SOD, PPO and peroxidase. There was no difference in banding patterns for the enzymes, esterase and SOD. Minor differences were observed for PPO and peroxidase. Low molecular weight bands were absent in few of the DH lines. The absence of those bands in DH lines cannot be attributed to any genetic cause since the parents and the respective hybrids were possessing the bands. Occurrence of isozymes is a general phenomenon in all organisms and they provide a natural marker system for investigating a variety of problems (Scandalios, 1974). Among the problems, analysis of genetic diversity in crop plants using the heterogeneity of isozymes is an important area. Electrophoretically identifiable isozymes have been utilised for the classification of rice varieties. Chu (1967), Sahai *et al.*(1969), Pai *et al.* (1973) and Fu and Pai (1979) showed the existence of peroxidase alleles specific to *indica* and *japonica* groups. Glaszman (1987) identified

six groups of rice varieties among 1688 traditional rices from Asia based on 15 polymorphic loci coding for eight different enzymes. However, the differences observed in the banding patterns of PPO and peroxidase were not distinct and the multiple forms of these enzymes could be due to a variety of factors such as occurance of artifact enzyme manipulations. Moreover, the isozyme polymorphisms observed based on the size differences couldnot be considered as ultimate evidences for answering the genetic differences (Scandalios, 1974). Isozymes can be used as potential markers, if the genetic cause for the occurance of multiple forms is well understood. Considering the above points, the utility of isozymes in the present study didnot establish a clearcut grouping of DH lines, hybrids and their parents.

The analysis of 22 genotypes using 30 random primers unravelled clearcut differences between the DH lines. The band sharing data of the RAPD markers established similarities between various pairs of DH lines as listed below:

i)	TNRH 16/30-6 and TNRH 16/30-7 (97 %).	
ii)	TNRH 19 and TNRH 19/1-37	(95.4%)
iii)	TNRH 19/8-56 and TNRH 21/6/60	(95.4%)
iv)	TNRH 19/1-37 and TNRH 19/1-48	(95.4 %)
v)	TNRH 19/1-48 and TNRH 19/8-55	(95 %)
vi)	TNRH 21 and TNRH 21/6-58	(96 %)
vii)	TNRH 21/6-60 and TNRH 21/8-68	(96.4%)
viii)	TNRH 21/6-60 and TNRH 21/8-70	(95.7%)
ix)	TNRH 21/8-68 and TNRH 21/8-70	(95.7%)
x)	IR 58025A and IR58025B	(95 %)

The minor differences existing between the DH lines can be attributed to so many factors. Heterogeneity in a DH population can be due to the heterozygous loci in the genome, which is true in the case of hybrids (Murigneux, 1993). Somaclonal variations can also cause variations among DH lines (Larkin and Scowcroft, (1981); Evans (1987)). In maize variation in banding pattern in tissue culture derived plants and seed grown plants had been reported by Brown *et al.*(1991). The occurrence of

non-parental banding profiles can also be explained due to the modification of the DNA during the anther culture process, either methylation changes or mutation. Another possibility for heterogeneity is the RAPD technique itself. RAPD markers often show distorted segregation and are difficult to reproduce, thereby creating problems when determining map locations (Kleinhofs *et al.*,1993.)

The dendrogram constructed based upon RAPD marker data (Fig-4) revealed the grouping of 22 genotypes into three diferent clusters .Cluster 1 had all the six DHlines and their hybrid TNRH16. The two other hybrids *viz.*,TNRH19 and TNRH21 and their DH lines were grouped into another cluster. In this cluster, the DH lines of TNRH19 and TNRH21 were grouped with their respective hybrids . The parents involved in the synthesis of all the three hybrids formed a separate cluster. The dendrogram also revealed the similarity between IR58025A and IR58025B. Among the random primers used one of the primers, *viz.*, OPF10 clearly differentiated the TNRH16 and its DH lines from all the genotypes involved in the study. Further, the RAPD analysis indicated that the variability prevailing among the DH lines of various hybrids was not of higher order as reported by Deepa (1999).

The RAPD markers used to assess the diversity of DH lines of three different hybrids revealed differences between groups of DH lines of each of the three hybrids. However, within the group differences were negligible and the dendrogram constructed based on the RAPD data revealed the possible differences between DH lines of TNRH16 and this could be due to the specific bands amplified by the primers *viz.*, OPU05-11 marker which is present only in TNRH16/3-5, OPF06-4, OPF06-6, OPF06-7 markers present only in TNRH16/3-5, OPAH18-1 marker present only in TNRH16/30-6, TNRH16/30-7 and TNRH16/33-11.

Inspite of low variability, among the RAPD markers amplified, unique banding pattern were observed for some of the genotypes studied. The primer OPF10 produced unique banding pattern in TNRH16 and the DH lines derived from it. OPF10-5 marker was present in IR58025A (female parent of hybrid), absent in C20R

(male parent of hybrid), present in the hybrid, but absent in all its DH lines. This shows the relation of the DH lines to the male parent. Similar pattern was noticed in the case of OPF10-11 marker for the same DH lines. OPF10-4 was a marker which differentiated TNRH16 and its derivatives from both its parents in that it was absent in both of the parents. A similar situation was noticed in the case of OPAH18-5 and OPAH18-6 markers which differentiated TNRH16 and its derivatives from both of its parents. The marker OPP2-1 was present in other two hybrids TNRH19, TNRH21, their derivatives and all the parents. But it was absent in TNRH16 and its derivatives. This is in accordance with the findings of Cao and Card (1997) in which unique banding patterns were found for the cultivars studied. Excluding these primers, no much difference was noticed in banding pattern among all the 22 genotypes studied.

Comparison of cluster analysis based on isozyme and RAPD markers showed that RAPD marker system has distinct advantages in fingerprinting DH lines. It is known that isozyme markers are limited in number and that they do not often reflect genetic relationships because of interactions with environment and varying expression at different growth stages.

In contrast, RAPD markers show genetic variation at the DNA level, allowing an estimation of the degree of relatedness between lines without the influence of environment. In this study, 30 random primers were used. If more detailed characterization is needed, more RAPD markers can be used to increase the number of markers.

In conclusion, the RAPD technique is a useful tool for fingerprinting very closely related rice DH lines in a more stable and reliable manner. This study also demonstrates that the DH lines developed at TNAU are quite different from each other, eventhough most of them have IR58025A as a common female parent. Thus, RAPD technique can be one of the methods in varietal identification, and genome composition in rice as well as in other crops.

SUMMARY

CHAPTER VI
SUMMARY

The present investigation was aimed at analysing the genetic diversity among the DH lines produced from rice hybrids *viz.*,TNRH16, TNRH19 and TNRH21, the hybrids and their respective parents. Isozyme and RAPD markers were used for the analysis. The results obtained are summarized below :

- Isozyme analysis involving four enzymes (esterase, SOD, PPO and peroxidase) did not reveal much difference between the genotypes involved. Esterase and SOD were monomorphic.
- PPO and peroxidase appeared to be polymorphic for some of the low molecular weight isoforms. But they were concluded to be artifacts as the corresponding isoforms were present in both the parents of respective DH lines.
- RAPD markers were highly polymorphic when compared to isozymes. The level of polymorphism observed for RAPD markers was 31.07 per cent.
- RAPD analysis gave clearcut differentiation of the DH lines based on the hybrid. Out of 22 genotypes, TNRH16 and its DH lines formed one cluster, TNRH19, TNRH21 and their DH lines formed second cluster. The third cluster possessed all the five parental genotypes.
- The results show that TNRH19 and TNRH21 are closely related.
- TNRH16 derived DH lines are more related to the male parent and this was clearly shown by the primers OPF10, OPAH18 and OPP02.
- There is variation within the DH lines in the case of TNRH16 as shown by the primers OPF06, OPU05 and OPU07. Among TNRH19 and TNRH21 derived DH lines no primer revealed such a type of variation. However, the variation observed between DH lines was not very high.
- RAPD is a reliable technique for fingerprinting closely related genotypes. It is more effective in differentiating individuals than isozyme markers.

REFERENCES

REFERENCES

Abe, T., Edanami, T., Adachi, E. and Sasahara, T. 1999. Phylogenetic relationships in the genus *Oryza* based on Mitochondrial RFLPs. **Genes and Genetic systems, 74 (1):** 23-27.

Beckmann, J.S. and Soller, M., 1990. Towards a unified approach to genetic mapping of eukaryotes based on sequence tagged microsatellite sites. **Biotechnology, 6**: 930-932.

Boso, G.S.C., Rangel, R.P.H and Ferreira, M.E. 1998. Analyses of genetic variability of South American rice Population (*Oryza glumeapatuta)* with isozymes and RAPD markers. **Molecular Ecology, 7 (1) :** 107 – 117.

Botstein, D., White, R., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphism. **Am. J. Hum. Genet., 32** : 314-331.

Briquet, M., Eloy, M.C. and Scheepers, D. 1996. Plant genetic biodiversity and molecular markers. **Agricontact No., 282 :** 17-21.

Brown, P.T.H., Gobel, E. and Lorz, H. 1991. RFLP analysis of *Zea mays* callus cultures and their regenerated plants. **Theor. Appl. Genet. 81** : 227-232.

Brown, P.T.H., Lange, F.D., Kranz, E. and Lorz, H. 1993. Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. **Mol. Gen. Genet., 237**: 311 – 317.

Brunk, C.F., Jones, K.C. and James, T.W. 1979. Assay for nanogram quatities of DNA in cellular homogenates. **Anal. Biochem., 92** : 497-500.

Burke, J.J. and Oliver, M.J. 1992. Differential temperature sensitivity of Pea superoxide dismutase. **Plant Physiol., 100** : 1595-1598.

Cai, H.W.X., Wang, K. and Chenj, K.S.1992. Specficity of expression for esterase isozyme gene in rice. **Acta Agriculture 18**: 392.

Camussi, A., Ottaviano, E., Calinski, T. and Kaczmarek, Z.1985. Genetic distances based on quantitative traits. **Genetics, 111** : 945-962.

Cao, D. and Oard, J. H. 1997. Pedigree and RAPD based DNA analysis of commercial US rice cultivars. **Crop Sci., 37** : 1630-1635.

Chan, C.Y., Young, C.T., Hwan, P.Y. and Soo, S.H. 1995. Genetic polymorphisms and phylogenetic relationships of Korean red rice (weedy rice in *Oryza sativa* L.) based on RAPD markers. **Korean J. Breed., 27** : 86-93.

Chu, Y. E. 1967. Variations in peroxidase isozymes of Oryza perennis and O. sativa. **Jpn. J. Genet. 42** : 233-240.

Chuan Qing, S., Long, L., Zhenshan,W., Li,Z. and Xiangkun, H.W. 1995. A primary study of cultivated rice *Oryza sativa* and common Chinese wild rice *O. rufipogon* using RAPD. **Chinese J. Rice Sci.,9** : 1-6.

Crouch, J.H., Crouch, H.K., Constandt H., Gysel, A.Van, Breyne, P., Montagu, M.Van, Jarret, R.L and Ortiz, R. 1999. Comparison of PCR based molecular marker analysis of Musa breeding populations. **Molecular Breeding 5(3)** 233-244.

Deepa, S. 1999. Genetic diversity analysis in doubled haploids of rice using RAPD analysis. M Sc thesis submitted to Tamil Nadu Agricultural University, Coimbatore-3.

Devos, K.M., Bryan, G.J., Collins, A. J., Stephenson, P. and Gale, M.D. 1995. Application of two microsatellite sequence in wheat storage proteins as molecular markers. **Theor. Appl. Genet. 90**: 247-252.

De Vries, H. 1912. **Species and varieties their origin by mutation.** Open Court Publ. Co., Chicago

Doebley, J.F.1989. Isozymic evidence and evolution of crop plants. In D.E Sottis and P.S Sottis (eds) **Isozymes in plant biology** Diosoorides press, Portland, pp 165-191.

Dubreuil, P, and Charcosset, A. 1999. Relatioships among maize inbred lines and populations from European and North American origins as estimated using RFLP markers. **Theor. and Appl.Genet. 99 (3/4):** 473-480.

Evans, D.A. 1987. Somaclonal variation. In : Nevins, D.J., Jones, R.A. (eds) **Tomato biotechnology**. (Plant biology Vol 4). Alan R.Liss, New York, pp.59-69.

Farooq, S., Shah, T.M., Arif, M. and Iqbal,N. 1995. Utilization of RAPD markers for identification of cultivated and wild rice species. **Pakistan J. Bot., 27** : 127-138.

Ford-Llyod, B.C., Jackson, M.T. and Newbury, H. J. 1997. Molecular makers and the management of genetic resources in seed gene banks a case study in rice. **CAB International** pp. 103-105.

Fu, P. Y. and Pai, C. 1979. Genetic studies on isozymes in rice plant.2. Classification and geographical distribution of cultivated rice through isozyme studies. **J. Agric. Assoc. China, 107** : 1-16.

Fukuoka, S., Hosaka, K. and Kamijima, O. 1992. Use of random amplified polymorphic DNA for identification of rice accessions. **Jpn. J. Genet., 84** : 567-572.

Glaszmann, J.C. 1985. A varietal classification of Asian cultivated rice *(Oryza_sativa* L) based on isozyme polymorphism. In. **Rice Genetics.**, IRRI, Philippines. pp 83-90.

Glaszmann, J.C. 1987. Isozymes and classification of Asian rice varieties. **Theor. Appl. Genetics. 74**: 21-30.

Glaszmann, J.C., de los Reyes, B.G. and Khush, G.S. 1988. Electrophoretic variation of isozymes in plumules of rice *(Oryza sativa* L) – a key to the identification of 76 alleles at 24 loci, **IRRI Research paper series, No:134:3-14.**

Godwin, I.B., Sangduen, N., Kunanuvatchaidach, R., Piperidis, G. and Adkins, S.W. 1997. RAPD polymorphisms among variant and phenotypically normal rice *(Oryza sativa* var *indica*) somaclonal progenies. **Plant Cell Reports, 16** : 320-324.

Gottlieb, L.D. 1981. Electrophoretic evidence and plant populations. In : **Progress in Phytochem.** Reinhold, L., Harborne, J.B., Swain, T. (ed) Vol.7, Oxford, Royanme-Uni, Pergamon Press, pp- 1-46.

Gottlieb, L.D. 1984. Electrophoretic evidence and plant population. **Progress in Phytochem., 7**: 1-46.

Grodzicker, T., Williams, J., Sharp, P. and Sambrook, K. 1974. Physical mapping of temperature sensitive mutations of adenoviruses. **Cold Spring Harbour Symp. Quant. Biol., 39** : 439-446.

Hakim, L. 1996. Detection of DNA sequence polymorphisms between mutant and mother variety of rice by RAPD. **J. Nuclear Agri. And Biol., 25** : 59-62.

Hilu, K.1984. The role of single gene mutations in the evolution of flowering plants. **Evol. Biol., 16 :** 97 – 128.

Huang, Z.L., Zhenshan, W., Hong, C., Ping, Y. and Xiangkun, W. 1996. Polymorphism of Chinese common wild rice (*O. rufipogon*) and cultivated rice (*O. sativa* L.) as determined by RAPDs. **J. Genet. Breed., 50** : 299- 307.

Ishikawa, R., Maeda, K., Harada, T., Minone, N and Saito, K. 1992. Genotypic variation for 17 isoenzyme genes among Japanese upland varieties in rice. **Jpn J.Breed., 42**: 737 – 746.

Jayaraman, K.S., Ramanuja, M.N., Vijayaraghavan, P.K. and Vaidhyanathan, C.S. 1987. **Food and Chemistry, 24**:203.

Kaneda, C., Umikawa, M., Singh, M.R., Nakamura, C. and Mori, N. 1996. Genetic diversity and subspecies differentiation in local rice cultivars fron manipur state of India. **Breeding Science, 46** : 159-166.

Kleinhofs, A., Kilian,A., Saghai Maroof, M.A., Biyashev, R.M., Hayes,P., Chen,F.Q., Lapitan,A., Fenwick,A., Blake,T.K., Kanazin,V., Ananiev,E., Dahleen,L., Kudrna,D., Bollinger,A., Knapp,S.J., Liu,B., Sorrells,M., Heun,M., Franckowiak,J.D., Hoffman,D., Skadsen,B., Steffenson,B.J. 1993. A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. **Theor. Appl. Genet. 86** : 705-712.

Ko, H.L., Cowan, D.C., Henry, R.J., Graham. G.C., Blakeney, A.B. and Lewin, L.G. 1994. RAPD analysis of Australian rice (*Oryza sativa* L.) varieties. **Euphytica, 80** : 179-189.

Larkin, P.J. and Scowcroft (1981). Somaclonal variation – a novel source of variability from cell cultures for plant improvement. **Theor. Appl. Genet. 60** : 197-214.

Lee, K.S., Senadhira, D. and Brar, D.S. 1996. Classification of Korean rice germplasm based on isozyme polymorphism. **International Rice Research Notes, 21:** 15.

Lee, M., Godshalk, E.B., Lamkey, K.R. and Woodman, W.W. 1989. Association of restriction fragment length polymorphisms among maize inbreds with agronomic performance of their crosses. **Crop Sci., 29** : 1067-1071.

Li Shuang Shen, He Ping, Xu Yun Bi, Tan Zen Bo, Lu Chao Fu and Zhu Li Huang. 1999. Genetic molecular linkage map construction and genome analysis of rice doubled haploid population. **Acta Botanica Sinica, 40** : 1115-1122.

Li Shuang, S. and Lihuang, Z. 1998. Direct PCR based genetic mapping of rice telomeric repeat associated sequences. **Genome, 4:** 193-198..

Martin, C., Julianu, A., New Bury, H,J., Lu, B.R., Jackson, M.T and Ford Lloyd, B.V. 1997. The use of RAPD markers to facilitate the identification of *Oryza sativa* within a germplasm collection. **Genetic Resources in Crop Evolution, 44(2):** 175 – 183.

Melody, S.C. 1997. Plant molecualr biology- A laboratory manual, Springer, Newyork.

Minobe, Y., Inoue, T., Zhang, H.S., Miyao, A., Ashikawa, I., Monna, L., Fukuoka, S., Miyadera, N., Nagamura, Y., Kurata, N. and Sasaki, T. 1994. Sequence tagged

sites as standard landmarkers in the rice genome. **Theor. Appl. Genet., 89** : 728-734.

Mishra, S.K., Asghar,M and Magpanfay, G.1996. Linkage between RFLP, isozyme and morphological markers in rice. **Indian J.Genet.Plant Breed., 56:** 366-369.

Monna Lasa, Alrio Miyao, Takakazn Inone, Shuichi Fuknoke, Muco Yamazaki, Hui Sun Zhong, Takuji Sasaki and Yuzo Minobe. 1994. Determination of RAPD markers in rice and their conversion into Sequence Tagged Sites (STSs) and STS-specific primers. **DNA Research, 1 (3):** 139-148.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via. a polymerase catalysed chain reaction. **Methods Enzymol., 155** : 335-351.

Murigneux, A., Barloy, D. Leroy, P. and Beckert, M. 1993. Molecular and morphological evaluation of doubled haploid lines in maize. 1. Homogeneity within DH lines. **Theor. Appl. Genet. 86** : 837-842.

Nakagahra, M. and Hayashi, K. 1976. Detection of esterase isozyme loci of *Oryza sativa* L. **Jpn.J.Breed.,26:** 114-115.

Oard, J.H. and Cao, D. 1997. Pedigree and RAPD based DNA analysis of commercial US rice cultivars. **Crop Sci., 37** : 1630-1635.

Oka, H.I.1958. Intervarietal variation and classification of cultivated rice. Indian **J.Genet Plant Breed, 18**: 79-89.

Oono, K. 1985. Genetic variability in rice plants regenerated from cell culture. **Cell and tissue culture for cereal crop improvement**. IRRI, Philippines, pp.95-104.

Pai, C., Endo, T. and Oka, H.I. 1973. Genetic analysis for peroxidase isozymes and their organ specificity in Oryza perennis and O. sativa. **Can. J. Genet. Cytol. 15** : 845-853.

Pai, C. and Fu, P.Y.1977. Geneic analyses for peroxidase and acid phosphatase isozymes in cultivated rice. **Agric.Bull. Taiwan, 2:** 75-85.

Parsons, B.J., Newburry, H.J., Jackson, M.T. and Ford–Lloyd, B.V. 1997. Contrasting genetic diversity relationships are revealed in rice *(Oryza sativa* L.) using different marker types. **Molecular Breeding, 3:** 115-125.

Patterson, H.D. and Weatherup, S.T.C. 1984. Statistical criteria for distinction between varieties of herbage crops. **J. Agr. Sci. (Camb), 102:** 59-68.

Perez, J.A., Maca, N. and Larruga, J.M. 1999. Expanding informativeness of microsatellite motifs through the analysis of heteroduplexes: a case applied to *Solanum tuberosum.* **Theor. Appl. Genetics, 99 (3/4):** 481-486.

Pham, J.L., Glaszmann, J.C., Sano, R., Barbier, P., Ghesquiere, A. and Second, G.1990. Isozyme markers in rice genetic analysis and linkage relationships. **Genome, 33**: 348-359.

Pisupati, B. 1999. Determining genetic diversity among *Porteresia coarctata* Tateoka accessions using morphological isozyme and RAPD markers. **Journal of Plant Biochemistry and Biotechnology, 8 (1)** : 21 – 24.

Qiang Wa, Liao Lanjie, Yang Dai chang, He Guang Cun and Shu Li Hui. 1998. RAPD analysis of wild rice genomes. **Journal of Tropical and Subtropical Botany 6(3):**260-266.

Qingming, Y., Wugo, D., Zongping, X. and Hua, P. H. 1995. Polymorphism and genetic relatedness among wild and cultivated rice species determined by AP-PCR analysis. **Hereditas, 122** : 135-141.

Raghunathachari, P., Khanna, V.K., Singh, N.K. and Singh, V.S. 1998. RAPD analysis of genetic variability in Indian scented 'Hansraj'rice germplasm. **Rice Biotechnology Quarterly. 35(5)**: 22 – 23.

Rao, V.R. and Riley, K.W 1994. The use of biotechnology for conservation and utilization of plant genetic resources. **Plant Genetic Resource News letter, 97**: 3-19.

Rohlf, F.J. 1992. **NTSYS-pc Numerical Taxonomy and Multivariate analysis system version 1.7.** Owner manual.

Romero, G.O., Amante-Bordeos, A.D., Dalmacio, R.K., Eilovan, R. and Sitch, L.A.1993. Comparitive studies of isozymes in *Oryza sativa, Oryza minuta* and their interspecific derivatives evidence for homology and recombination. **Theor. Appl. Genet., 87**: 609-615.

Sadasivam, S. and Manickam, A. 1996. **Biochemical methods**. Wiley Eastern Ltd. And Tamil Nadu Agricultural University, Coimbatore.

Sambrook, J., Fristsch, E.F., Maniatis, T. 1989. **Molecuar cloning: a laboratory manual** 2nd ed. Cold Spring Harbor NY, USA.

Sane, A.P., Seth, P.; Rannade, S.P., Nath, P. and Sane, P.V. 1997 RAPD analysis of isolated mitochondrial DNA reveals heterogeneity in elite wild abortive (WA) cytoplasm in rice. **Theor. Appl. Genetics, 95 (7):** 1098-1103.

Scandalios, J.G. 1974. Isozymes in development and differentiation. **Ann. Rev. Plant Physiol., 25** : 225-258.

Schut, J.W.; Qi, X and Stam, P. 1997. Association between relationship measures based on AFLP markers, pedrgriee data and morphological traits in barley **Theor. Appl. Genetics 95** (7): 1661-1168.

Second, G. 1985. Evolutionary relationships in the *sativa* group of *Oryza* based on isozyme data. **Genet. Sel. Evol., 17**: 89-114.

Second, G.1991. Molecular markers in rice systematics and evaluation of genetic resources. In: Bajaj YPS (ed) **Biotechnology in agriculture and foresty. Vol. 14**, Rice Springer Verlag, Berlin Heidelberg, pp: 468 – 494.

Sahai, B.B., Morishima, H. and oka, H.I. 1969. A survey of variations in peroxidase, acid phosphatase and esterase isozymes of wild and cultivated *Oryza* species. **Jpn. J. Genet. 44** : 303-319.

Siddiq, E.A. 1999. **The Hindu Survey of Indian agriculture**. pp: 39-46.

Smith, H.H., Hamil, D.E., Waever, E.A., and Thompson, K.H. 1970. Multiple molecular forms of peroxidases and esterases among *Nicotiana* species and amphidiploids. **J.Hered., 61**: 203-212.

Smith, J. A. and Hammerschmidt, R. 1988. Comparative study of acidic peroxidases associated with induced resistance in cucumber, muskmelon and water melon. **Phsiol. Mol. Plant Pathol., 33** : 255- 261.

Smith, J. S. C. 1988. Diversity of United States hybrid maize germplasm : Isozymic and chromatographic evidence. **Crop Sci., 28** : 63-69.

Souza, E. and Sorrells, M.E. 1989. Pedigree analysis of North American oat cultivars released from 1951 to 1985. **Crop Sci., 29** : 595-601.

Stuessy, T. 1990. **Plant taxonomy.** Columbia Unico Press, New York.

Stuber, C.W.1992. Biochemical and molecular markers in plant breeding. In. Janick J.Wiley J and Sons (eds). **Plant Breeding Reviews, Vol. 9**, John Wiley and Sons, Chichester Brisbane, pp: 37-61.

Sun Xinhi, Cai Hong Wei and Wang Xiang Kun. 1996. A method for studying electrophoretic variation of isozymes in rice with polyacrylamide gel. **Chinese J. Rice Sci., 10:** 43-50.

Sun, H.S., Sato, Y.I. and Morishima, H. 1997. Genetic characterization of weedy rice (*Oryza sativa* . L) based on morphology. Isozymes and RAPD markers. **Theor. Appl. Genet., 94 :** 316 – 321.

Sun, Z.X., Zhao, C.Z., Zheng,K.L., Qi, X.F., Fu, Y.P. 1983. Somaclonal genetics of rice Oryza sativa L. **Theor. Appl. Genet. 67** : 67-73.

Tanksley, S.D., Young, N.D., Paterson, A.H. and Bonierbala, M.W. 1989. RFLP mapping in plant breeding. New tools for an old science. **Biotechnology, 7**: 257-264.

Toenniessen, G.H. and Khush, G.S. 1991. Prospects for the future. In: **Rice Biotechnology.** Ed. Khush, G.S. and Toenniessen, G.H. IRRI, Philippines. pp.309-313.

Tragoonrung, S., Kanazin, V., Hayes, P.M. and Blake, T.K. 1992. Sequence tagged site facilitated PCR for barley genome mapping. **Theor. Appl. Genet., 84** : 1002-1008.

Virk, P.S., Ford Lloyd, B.V., Jackson, M.T. and New Bury, H.J.1995. Use of RAPD for the study of diversity within plant germplasm collections. **Heredity, 74 (2):** 170 – 179.

Virk, P.S., Ford-Llyod, B.V., Jackson, M.T., Poom, H.S., Clemeno, T.P. and Newbury, H.J. 1996. Predicting quantitative variation with in rice germplasm using molecular marker . **Heredity 76:** 296 – 304.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. **Nucleic Acid Research, 18 :** 6231 – 6235.

Winter, P. and Kahl, G. 1995. Molecular marker technologies for plant improvemt. **World J.Microbiol. Biotech., 11:** 438 – 448.

Wu, K.S, Glaszmann, J.C. and Khush, G.S. 1988. Chromosomal location of ten isozyme loci in rice (*Oryza sativa* L) through trisomic analysis. **Biochem. 26:** 303-320.

Xiao, J., Li, J, Yuan, L., McCouch, S.R. and Tanksley, S.D. 1996. Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR – based markers. **Theor.Appl. Genetics, 92:** 637-643.

Xu Liu Dong and Xian Li Zi. 1995. Identification of distant hybrid with the characteristic bands of esterase isozyme of the leaves in rice (Oryza). **Journal of South East Agrl. University,** 17: 386-390.

Xuan Jun Fang, Huang Yu Ming, Chen Qi Feng and Sun Mei. 1999. Genetic analysis of some elite rice cultivars and hybrid rice combinations by using allozyme and RAPD markers. **Scientia Agricultural sinicia, 32 (2)** : 1-8.

Yu, L.X. and Nguyen, H.T. 1994. Genetic variation detected with RAPD markers among upland and lowland rice cultivars *(Oryza sativa* L.). **Theor. Appl. Genet.,** 87: 668-672.

Zhou, Z. and Gustafson, J.P,1995 a. Genetic variation detected by DNA fingerprinting with a rice minisatellite probe in *Oryza sativa* L. **Thoer. Appl. Genet.**, **91** : 481-488.

Zhou, Z. and Gustafson, J.P. 1995b. Characterization of minisatellite sequences in rice and their applications for DNA fingerprinting. **Proceedings on induced mutations and molecular techniques for crop improvement** pp 205-214.

Zhu, J., Gale, M.D., Quarrie, S., Jackson, M.T. and Bryan, G.J. 1998. AFLP markers for study of rice biodiversity. **Theor. Appl. Genet., 96** : 602-611.

Zvingila, D., Klezaite, V. and Popendikyte, U. 1998. Analysis of RAPD and isozyme (SOD) polymorphism in cereals. In **Biotechnologija augalu selek cijoje**. Moksline Koneferencija, lieturos Zemes Ukio Universitetas.

APPENDIX

APPENDIX I

Hoagland's Solution

Solution I

Calcium nitrate	:	5.906 g in 100 ml

Solution II

Potassium nitrate	:	2.528 g
Magnesium sulphate	:	2.466 g
Potassium dihydrogen phosphate	:	3.41 g
Dissolved in 100 ml water		

Solution III

EDTA (Sodium salt)	:	1.75 g
Ferrous sulphate	:	2.466 g
Dissolved in 100 ml water		

Solution IV

Boric acid	:	0.205
Manganese sulphate	:	0.155g
Copper sulphate	:	0.025g
Sodium molybdate	:	0.005g
Zinc sulphate	:	0.005g
Cobalt chloride	:	0.005g

All solutions were autoclaved. 5ml each of solution I, II and IV and 3 ml of solution III were taken and volume was made upto 1000ml and used for watering the seedlings.