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Full Length Research Paper

Genetic diversity study of important Indian rice genotypes using biochemical and molecular markers

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Rice is a staple food for 90% of the world. Genetic characterization of natural resources is an essential step to understand genetic resources. In the present study, commonly using 25 Indian rice genotypes were collected / procured from four different states of India. Genetic variation was assessed using isozyme and RAPD (random amplified polymorphic DNA) markers. A total of 25 isozyme putative loci and 273 RAPD bands were generated with 64 and 96% polymorphism respectively were obtained. The genetic similarity coefficient of isozyme study calculated from Jaccard's similarity coefficient ranged from 0.702 to 0.95 where as the genetic similarity coefficient of RAPD study calculated from Jaccard's similarity coefficient ranged from 0.328 to 0.806. Like the previous study the present indicates that isozyme and RAPD markers are reliable, efficient and effective techniques to determine the genetic variation in rice.

Key words: Genetic diversity, isozyme, RAPD, rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important food crops grown worldwide and is the staple food for half of the world population (Sasaki and Burr, 2000). Rice is grown under diverse eco-geographical conditions in various tropical and subtropical countries, including India. To meet the future demand for food, anticipated from the projected world population increase, there is an urgent need to take all necessary steps to enhance the productivity of this crop.

The quality preferences of rice consumers have resulted in a wide diversity of varieties specific to different localities. Although the exact diversity cannot be gauged, it is estimated to be around 140 000 different genotypes. The IRRI gene bank preserves nearly 100,000 accessions. India alone has 86,330 accessions, of which 42,004 are in the national gene bank (Rai, 1999), which is enriched by further explorations, collections and conservation. Sociocultural traditions have increased the diversity of Indian rices in terms of morphological and quality traits, especially grain size, shape and colour, as well as aroma and endosperm properties. Ancient *Ayurvedic* literature (Indian *Materia Media*) from the fifteenth and sixteenth centuries A.D. describes different rices, particularly scented varieties with medicinal and curative properties. As far back as 400 B.C., *Susruta*, the great Indian pioneer in medicine, described the medicinal properties of rice.

India is a primary centre of origin of rice and has many local landraces, most of which are not cultivated today. While many are lost, a few are still cultivated by resource-poor traditional farmers in areas practicing subsistence farming. The exact genetic potential, differences from commercial varieties, and the magnitude of heterogeneity still present in local landraces are not well catalogued. The need to characterize available landraces has therefore become important in modern crop improvement

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Abbreviations: EST, Esterase; **AEST,** acetyl esterase; **AMY,** α–amylase; **CAT,** catalase; **ACP,** acid phosphatase; **ALP,** alkaline phosphatase; **AOD,** aldehyde oxidase; **XDH,** xanthine dehydrogenase; **JR,** Jarkhand; **MH,** Maharashtra; **TN,** Tamilnadu; **WB,** West Bengal; **RFLP,** restriction fragment length polymorphism; **RAPD,** random amplified length polymorphism; **SSRs,** simple sequence repeats; **ISSRs,** inter-simple sequence repeats; **AFLP,** amplified fragment length polymorphism; **SNPs,** single nucleotide polymorphisms; **CTAB,** cetyltrimethylammonium bromide; **EDTA,** ethylene diamine tetraacetic acid; **TE,** tris-EDTA.

(Frey et al., 1984; Dale et al., 1985; Rezai and Frey, 1990). Moreover, rice is also an ideal model plant for the study of grass genetics and genome organization due to its diploid genetics, relatively small genome size 430 Mb, significant level of genetic polymorphism (McCouch et al., 1998; Wang et al., 1992), large amount of well conserved genetically diverse material (approximately 100,000 accessions of rice germplasm worldwide) and the availability of widely collected, compatible wild species.

Molecular markers have demonstrated a potential to detect genetic diversity and to aid the management of plant genetic resources (Ford-Lloyd et al., 1997; Song et al., 2003). In contrast to morphological traits, molecular markers can reveal differences among genotypes at the DNA level, providing a more direct, reli-able and efficient tool for germplasm characterization, conservation and management. Different types of mole-cular markers have been used to investigate the bio-diversity of rice (Glaszmann, 1987; Wang and Tanksley, 1989; Wu and Tanksley, 1993; Zeitkiewicz et al., 1994; Virk et al., 1995; Zhou and Gustafson, 1995; Ashikawa et al., 1999; Dong et al., 2000).

Characterization and quantification of genetic diversity has long been a major goal in evolutionary biology. Information on the genetic diversity within and among closely related crop varieties is essential for a rational use of genetic resources. The analysis of genetic variation both within and among elite breeding materials is of fundamental interest to plant breeders. It contributes to monitoring germplasm and can also be used to predict potential genetic gains.

Morphological characteristic might themselves be insufficient to distinguish between pairs of closely related species geographical races, or ecotypes, because not allgenetic differentiation results in morphological differentiation (Siva and Krishnamurthy, 2005). Thus, a genetic characterization of natural resources is an essential step for a better understanding of genetic resources for the implementation of in situ and ex situ conservation activities (NBPGR, 2000). So adequate knowledge about the plants is necessary for planning sustainable development of any region like India, where the flora is rich in diversity and endemism.

The objectives of present study were to use Isozyme and RAPD markers to evaluate the genetic variation within a diverse collection of twenty five rice accessions, to determine differences in the patterns of diversity to distinguish different accessions, and to reveal the genetic relationships among them.

MATERIALS AND METHODS

Plant materials

Seeds of 25 rice genotypes were obtained from different parts of Jharkhand, Maharashtra, Tamil Nadu and West Bengal, (Table 1). The seeds were sown in polycarbonate pots to raise seedlings.

Isozyme analysis

Young leaves were homogenized in two volumes of cold (4°C) extraction buffer containing 0.1 M Tris-HCl, pH 7.2, 5% Sucrose, 0.5% (w/v) PVP, 10 mM mercaptoethanol. The samples were then centrifuged at 15,000 rpm for 15 min at 4°C, supernatant was collected in a separate vial. Each sample was applied to a native discontinuous polyacrylamide gel (6% stacking, 8% separating gel) and the runs were performed on a mini gel apparatus in Tris-glycine (pH 8.3) buffer. Nine enzyme systems were examined viz., aldehyde oxidase (ADH – EC. 1.2.3.1), acid phosphatase (ACP – EC.3.1.3.2), alkaline phosphatase (ALP – EC. 3.1.3.1), α –amylase (AMY – EC. 3.2.1.1), acetyl esterase (AEST – EC 3.1.1.6), catalase (CAT – EC. 1.1.1.48), esterase (EST – EC. 3.1.1.2), nicotinamide adenine dinucleotide diaphorase (EC 1.8.1.4.) and xanthine dehydrogenase (EC 1.17.1.4.)

Genomic DNA extraction

DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). For each accession, about 1 g of bulked leaf tissue homogenized in 6 mL of extraction buffer (20 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 2% CTAB, and 1% β-mercaptoethanol) was added. The suspension was mixed well, incubated at 60°C for 45 min, followed by chloroform – isoamyl alcohol (24:1) extraction and precipitation with 2/3 of the volume of isopropanol at –20°C for 1 h. The DNA was pelleted down by centrifugation at 12 000 rpm for 10 min and suspended in TE buffer (10 mM Tris-HCl – 1 mM EDTA (pH 8.0)). The DNA was purified from RNA and proteins by standard procedures (Sambrook et al. 1989) and DNA concentration was estimated by agarose gel electrophoresis and staining with ethidium bromide.

PCR amplification

A set of 25 random primers (Bangalore Genei, India) were screened. Polymerase chain reaction (PCR) was performed in 25 µl reaction mixture reaction containing 10X Taq buffer A (Genei, India) containing Tris with $MgCl_2$, 10mM dNTP mix, RAPD primer (100ng/µl), DNA template (10ng/µl), Taq DNA Polymerase (3U/ µl). Amplifications were performed in a Minicycler (MJ Research). The thermal profile of PCR was 40 cycles at 94°C, 45s; 35°C, 60 s and 72°C, 90 s. Before the first cycle, the temperature of 94°C was maintained for 5 min and after the last cycle 72°C was maintained for 10 min. On completion of PCR, 8 µl of PCR products were resolved in 1.5% agarose(SRL) gels stained with ethidium bromide using 1X TBE buffer in a submarine gel apparatus at 100 V. The gels were visualized under ultraviolet light, and photographed in a gel documentation system. All reactions were performed at least twice.

Gel scoring and data analysis

All the genotypes were scored for the presence and absence of the SSR bands. And the data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. The Excel file containing the binary data was imported into NT Edit of NTSYS-pc 2.02J. The 0/1 matrix was used to calculate Similarity as DICE coefficient using SIMQUAL subroutine in SIMILARITY routine. The resultant similarity matrix was employed to construct dendrograms using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means (UPGMA) to infer genetic relationships and phylogeny.

Table 1. Isozyme analysis of rice varities.

Variety	State	EST	AEST	AMY	CAT	ACP	ALP	AOD	XDH	A	P
ADT 37	TN	0.75	1	0.5	0.66	0.4	0.5	1	1	0.68	52.70
ADT 43	TN	0.75	1	0.25	0.66	0.4	1	1	1	0.72	62.37
BPT	MH	0.75	1	0.5	0.66	0.6	1	1	1	0.76	54.54
BN 20	WB	1	1	0.25	0.33	0.4	0.5	1	1	0.64	49.58
Hansa	MH	1	0.50	0.25	0.66	0.6	1	1	1	0.68	60.01
HMT	MH	1	0.50	0.50	0.66	0.4	1	1	1	0.68	51.66
IR 64	WB	1	0.5	0.5	0.66	0.6	0.5	1	1	0.68	57.91
IR 36	JR	0.75	1	0.25	0.33	0.8	1	1	1	0.72	61.70
IET 9947	WB	0.75	1	0.5	0.66	1	1	1	1	0.84	67.94
JJL	MH	0.75	1	0.75	0.66	0.8	1	1	1	0.84	68.20
J36	MH	0.75	1	0.75	0.66	0.6	0.5	1	1	0.76	61.45
1001	MH	1	1	0.75	0.33	0.6	0.5	1	1	0.76	69.43
MTU1010	WB	1	0.75	0.75	0.33	0.6	0.5	1	1	0.8	65.84
HMT Sona	TN	0.75	1	0.50	0.33	0.8	1	1	1	0.76	59.87
New 1010	MH	0.75	1	0.75	0.66	1	1	1	1	0.88	69.70
Lakhansal	WB	1	1	0.75	0.33	0.8	0.5	1	0	0.72	59.91
Kshitish	WB	1	1	0.75	0.33	1	1	1	0	0.8	59.66
N.C.Kalsa	WB	0.75	0.5	0.5	0.66	0.8	0.5	1	1	0.68	67.29
Ponni	TN	1	1	0.5	0.66	1	0.5	1	1	0.84	55.41
Sonalika	MH	0.75	0.5	0.5	0.66	0.88	1	1	1	0.72	43.54
Sonamasuri	MH	1	1	0.5	0.66	0.8	0.5	1	1	0.8	62.91
Swati	MH	0.75	0.5	0.25	0.66	0.8	1	1	1	0.68	59.37
Swarna	WB	0.75	0.5	0.75	0.66	1	0.5	1	1	0.76	43.95
1010	WB	1	0.5	0.75	1	1	0.5	1	1	0.84	67.25
Pankaj	JR	0.75	0.5	0.75	0.66	1	0.5	1	1	0.76	53.95

EST, Esterase; AEST, acetyl esterase; AMY, α – amylase; CAT, catalase; ACP, acid phosphatase; ALP, alkaline phosphatase; AOD, aldehyde oxidase; XDH, xanthine dehydrogenase, JR, Jarkhand; MH, Maharashtra; TN, Tamilnadu; WB , West Bengal.

RESULTS AND DISCUSSION

The greatest enzymatic activities were obtained from fresh leaves. The centrifuged extracts were used immediately in order to avoid loss in enzymatic activity. The 25 Putative allelic loci were obtained from the above said different enzyme systems. Among the nine enzymes, acid phosphatase yielded the highest number of alleles (Electromorphs/bands) as five, followed by EST, AEST and amylase with four bands each. While, catalase with three bands and alkaline phosphatase and xanthine dehydrogenase has two bands each. Single band was observed in aldehyde oxidase.

Data on loci shared for each variety and allele frequency for all varieties are given in Table 1. Out of 25 alleles, 9 alleles were known to found in all 25 varieties. They were EST- 1, EST-3, EST-4, AEST- 1, AEST- 4, CAT- 1, ACP- 1, ALP- 1, and ADO- 1. The variety New 1001, consisted of the maximum number of loci, i.e 22 and its allele frequency was 0.88, followed by varieties IET 9947, Ponni ,1010 and JJL with 21 with an allele frequency of 0.84in each case. MTU 1010, Kshitish and Sonamasuri had 20 alleles with an allele frequency of 0.8 in each case. Pankaj, Swarna, HMT Sona, J36, 1001 and BPT had 19 alleles with their allele frequency of 0.76 in each case. ADT 43, IR 36, Lakhansal and Sonalika had 18 alleles with their allele frequency of 0.72. ADT 37, Hansa, HMT, IR 64, Swati and N.C Kalsa had 17 alleles with an allele frequency of 0.68. Of all varieties BN-20 had least number of 16 loci with an allele frequency of 0.64. All Varieties of *O. sativa* were monomorphic with reference to aldehyde oxidase.

The UPGAMA dendrogram in Figure 1 shows how the accessions are grouped on the basis of Jaccard Coefficients. The first two branches separate ADT 37 and IR 64, and ADT 43 from the other accessions. There are two main two clusters: Hansa, HMT, Swati, N.C.Kalsa, Sonalica, Swarna, 1010 and Pankaj as one group and ADT 43 to MTU 1010 as a second cluster consisting of 15 accessions.

In the case of RAPD analysis, we obtained 273 bands from all populations. Of all bands observed 96% were polymorphic among all populations. The number of bands generated by each primer ranged from 6 to 20. All primers generated polymorphic bands. All primers except RPI 7, RPI 10, RPI 12, RPI 13, RPI 17, RPI 18 and RPI

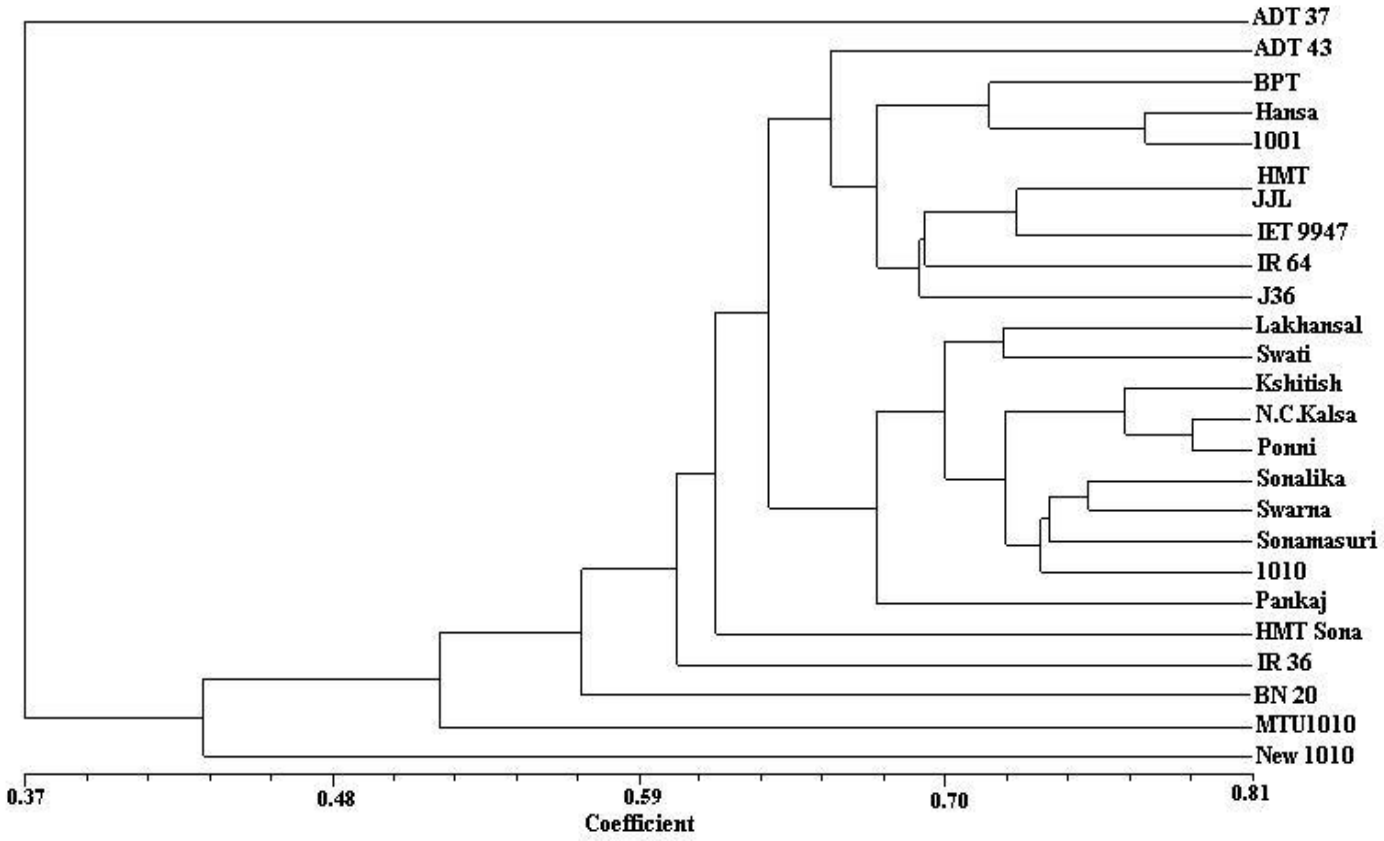

Figure 1. Dendrogram showing clustering of 25 Indian rice genotypes constructed using UPGMA based on Jaccard's coefficient obtained from RAPD analysis.

20 revealed 100% polymorphism. Most of the band size ranged between 100 to 1000 bp, with an average of 13.6 bands per primer. The genetic similarity coefficient calculated from RAPD study ranged from 0.328 to 0.806.

A survey of 25 accessions of O. sativa using isozyme and RAPD markers indicated that RAPD markers are able to disclose a much higher level of polymorphism than revealed by isozymes, essentially at the intraspecific level. In fact, most isozymes were monomorphic, while a high level of variation was detected for RAPDs. Genetic diversity and distance derived from isozyme analyses were very low and due to small number of polymorphic alleles, any small difference had a strong influence on the distribution and grouping of accessions.

In our study, both methods of analysis demon-strated that the collected accessions are closely related based on either the states from where it has been collected or based on their parental types (Figure 2). For example, Sonalika and Swarna were from Maharashtra state and showing the affinity. In a similar way, Pankaj and var.1010 were from West Bengal and indicating the similar affinity; this indication is very clear, even though the two approaches yielded a different clustering of accessions and therefore, slightly different dendrograms.

India is the second-largest rice-producing country in the world, where about 60000 landraces were being grown in the 1950s (Anonymous 1992). Until 1968, the varieties deve-loped by agricultural research stations in India were largely by direct pureline selection from landraces and occasionally derived by selection after hybridization between purelines.

Such varieties are referred to as traditional cultivars in this paper. Later on, modern culti-vars were developed by hybridization of the local land-races and traditional cultivars with exotic germplasm. The present study included 25 accession collected from four different states of India.

Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species. Several molecular markers viz. Restriction fragment length polymorphism (RFLP) (Becker et al., 1995; Paran and Michelmore, 1993), random amplified length polymorphism (RAPD) (Tingey and Deltufo, 1993; Williams et al., 1990), simple sequence repeats (SSRs) (Levinson and Gutman, 1987), inter-simple sequence repeats (ISSRs) (Albani and Wilkinson, 1998; Blair et al., 1999), amplified fragment length polymorphism (AFLP) (Mackill

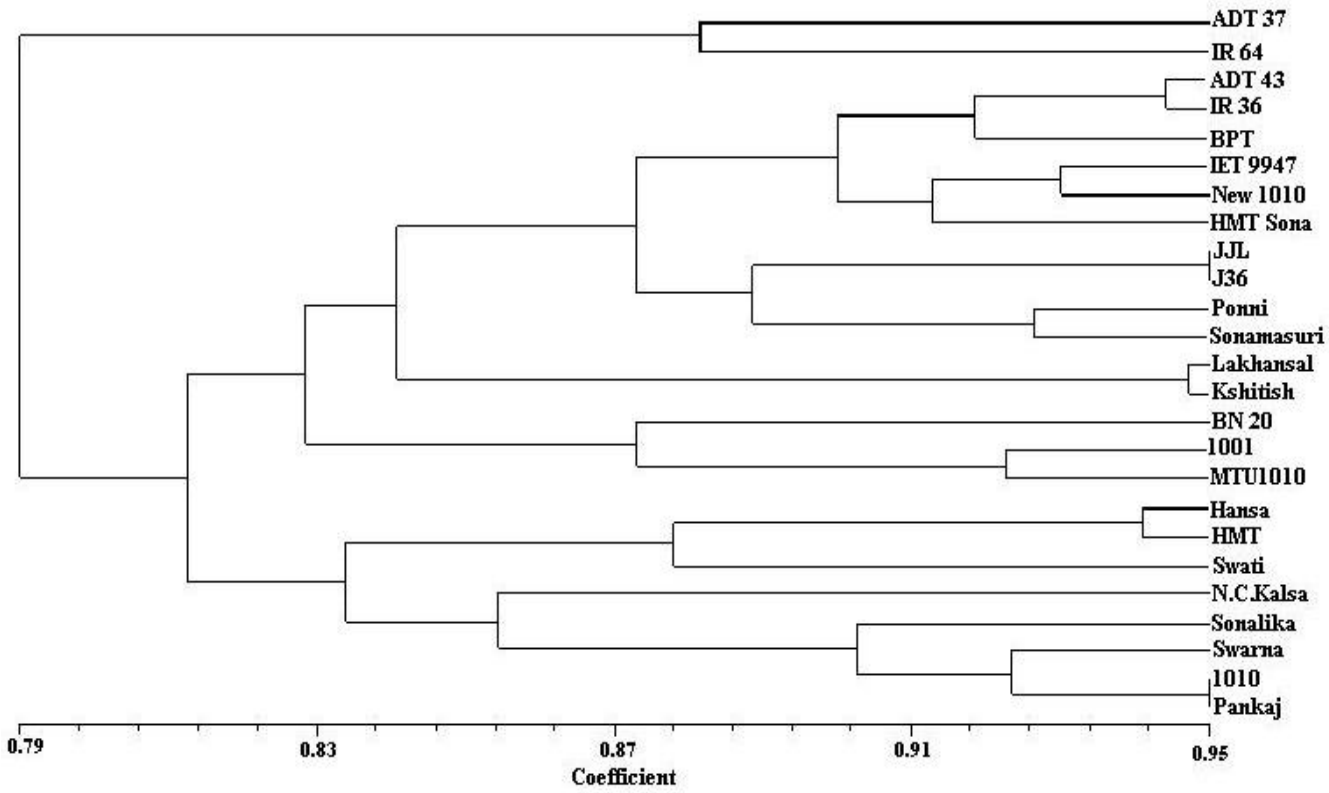

Figure 2. Dendrogram showing clustering of 25 Indian Rice genotypes constructed using UPGMA based on Jaccard's coefficient obtained from Isozyme analysis.

et al., 1996; Thomas et al., 1995; Vos et al., 1995; Zhu et al., 1998) and single nucleotide polymorphisms (SNPs) (Vieux,et al., 2002) are presently available to assess the variability and diversity at molecular level (Joshi et al., 2000). Information regarding genetic variability at molecular level could be used to help, identify and develop genetically unique germplasm that compliments existing cultivars**.**

Modern cultivars showed more diversity than the landraces from the same region, and yet clustered closer to the traditional cultivars. Therefore, these results indicate that the development of modern cultivars does not appear to have caused genetic erosion. In fact, after hybridization of the landraces and their derivatives with the exotic germplasm, genetic diversity has increased in terms of available polymorphisms at single loci. However, in terms of cultivated genotypes, the total genetic diversity erosion is probable, because only a small number of the 500 modern cultivars (Anonymous 1992) are currently being cultivated in India, and they occupy more than 95% of the area under rice cultivation. Therefore, it remains essential to conserve the biodiversity present in the rice landraces and use it to broaden the genetic base of cultivated rice varieties. Molecular markers, such as isozymes and RAPD will play an essential role in characterizing biodiversity for its exploitation in modern breeding programmes.

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