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Original article

Genetic diversity among cultivars, landraces and wild relatives of rice as revealed by microsatellite markers

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Abstract. Genetic diversity among 35 rice accessions, which included 19 landraces, 9 cultivars and 7 wild relatives, was investigated by using microsatellite (SSR) markers distributed across the rice genome. The mean number of alleles per locus was 4.86, showing 95.2% polymorphism and an average polymorphism information content of 0.707. Cluster analysis based on microsatellite allelic diversity clearly demarcated the landraces, cultivars and wild relatives into different groups. The allelic richness computed for the clusters indicated that genetic diversity was the highest among wild relatives (0.436), followed by landraces (0.356), and the lowest for cultivars. Allelic variability among the SSR markers was high enough to categorize cultivars, landraces and wild relatives of the rice germplasm, and to catalogue the genetic variability observed for future use. The results also suggested the necessity to introgress genes from landraces and wild relatives into cultivars, for cultivar improvement.

Keywords: genetic diversity, rice landraces, microsatellite markers, multiple alleles, *Oryza* spp., PIC values.

Introduction

Rice (*Oryza sativa* L.) is the principal staple food for more than 50% of the world's population. 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. By the end of the 1980's, the exploitable genetic yield potential through conventional breeding approaches had been achieved and yield started plateauing even in experimental trials. The increased frequency of drought in arable regions threatens rice production and demands the development of rice genotypes capable of producing more from diminishing water resources. Additionally, rice improvement efforts should also be directed towards: incorporation of resistance to diseases and insects; reduction of growth duration; and improvement of grain quality. To address these problems, it is essential to search for new traits among the wild genetic resources and landraces.

Landraces harbour a great genetic potential for rice improvement. Unlike high-yielding varieties (whose variability is limited due to homozygosity), the landraces maintained by farmers are endowed with tremendous genetic variability, as they are not subjected to subtle selection over a long period of time. This aids in the adaptation of landraces to wide agro-ecological niches and they also have unmatched qualitative traits and medicinal properties. This rich variability of complex quantitative traits still remains unexploited. Landraces are also important genetic resources for resistance to pests and fungal diseases. For instance, Indian landraces Velluthachira, Bengle and Bhumansam are resistant to rice gall midge, Chemban is resistant to brown plant hopper, Tadukan is resistant to blast, while Buhjan and Laka are resistant to sheath blight (Siddiq et al.

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2005). Owing to their specific domination in geographical niches, landraces have genes of resistance to abiotic stresses, which have not been widely utilized or incorporated into modern varieties. For example, some of the South Indian rice landraces, like Norungan and Noortripathu, are now used as donors for drought tolerance (Ganesh et al. 2004). Collection and evaluation of landraces are an integral part of the pre-breeding process carried out by rice breeders (Vaughan 1991; Siddiq et al. 2005).

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 is not well catalogued. The need to characterize available landraces has therefore become important in modern crop improvement (Frey et al. 1984; Dale et al. 1985; Rezai and Frey 1990).

Wild relatives of cultivated rice also play a vital role in rice breeding (Vaughan 1994). A good example of this is the large-scale adoption of hybrid rice technology, which originated from the successful transfer of the male sterility gene from *O. rufipogon* Griff. to produce cytoplasmic genetic male-sterile (CMS) lines. This technology increased rice production by about 10–20% over the past 20 years in China (Yuan 1993). The first cloned rice disease-resistance gene, *Xa21*, encoding a protein with unusual leucine-rich-repeat (LRR) kinase domains (Song et al. 1995), was introgressed from a wild rice species, *O. longistaminata*. This gene confers a broad spectrum of resistance to bacterial blight disease in rice (Zhai et al. 2000).

Molecular markers have demonstrated a potential to detect genetic diversity and to aid the management of plant genetic resources (Ford-Lloyd et al. 1997; Virk et al. 2000; Song et al. 2003). In contrast to morphological traits, molecular markers can reveal differences among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management. Several types of molecular markers are available today, including those based on restriction fragment length polymorphism (RFLP) (Botstein et al. 1980), random amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al. 1990), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), and simple-sequence repeats (SSRs) or microsatellite markers (Tautz 1989). Of these, RFLP and microsatellite markers are codominant and their map positions on the rice genome are well known, while RAPD and AFLP markers produce random amplification and are largely dominant markers. Microsatellites are PCR-based markers that are both technically efficient, cost-effective, and common in rice (Chen et al. 1997; Temnykh et al. 2000). Compared to RFLPs, microsatellite markers detect a significantly higher degree of polymorphism in rice (Wu and Tanksley 1993; Yang et al. 1994), and are especially suitable for evaluating genetic diversity among closely related rice cultivars (Akagi et al. 1997). The present study addresses the utility of SSR markers in revealing genetic relationships at the molecular level among local cultivars, landraces and wild species of rice collected from a wide range of agro-geographical regions of the Indian subcontinent.

Materials and methods

Plant materials

Seeds of 35 rice genotypes (19 landraces, 9 cultivars and 7 wild relatives), representing all 3 major components of rice germplasm (Table 1 and Figure 1), were obtained from the Paddy Breeding Station, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, India. The seeds were sown in polycarbonate pots to raise seedlings.

DNA extraction and SSR analysis

Total DNA was extracted from fresh leaves by the cetyl tri-methyl ammonium bromide (CTAB) method (Murray and Thompson 1980). The quality and concentration of extracted DNA were estimated by using a UV-Vis spectrophotometer. For SSR analysis, a total of 25 SSR primer pairs obtained from Research Genetics Inc. (USA) were used for PCR amplification (Table 2). PCR conditions were maintained as described by Panaud et al. (1996). Each PCR reaction was carried out in a 15 μL reaction volume containing 0.2 μM of each primer, 200 μM of deoxyribonucleotides, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatine, 40 ng of DNA, and 0.5 unit of Taq DNA polymerase. The temperature profile used for PCR amplification comprised 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and ending up with 5 min at 72°C for the final extension. The anneal-

Table 1. Genotypes used in SSR analysis, their sites of collection, and special traits

Sl. no.	Accession	Site of collection	Special traits
1	Periavellai	Ceylon	leaf folder tolerance
2	Katta	Tanjore, Tamil Nadu	stem borer tolerance
3	Hroujothi	Chenglepet, Tamil Nadu	stem borer tolerance
4	Gandaasali	South Canara, Karnataka	stem borer and leaf folder tolerance
5	Garika	Chitoor, Andhra Pradesh	blast tolerance
6	Sirumani	Chenglepet, Tamil Nadu	stem borer tolerance and better grain quality
7	Koolavalai	Tirunelveli, Tamil Nadu	stem borer, leaf folder and blast tolerance
8	SadaiSamba	Coimbatore, Tamil Nadu	stem borer, leaf folder and brown plant hopper (BPH) tolerance
9	KalarSamba	Salem, Tamil Nadu	leaf folder and bacterial leaf blight (BLB) tolerance
10	AruamKuruvai	Tirunelveli, Tamil Nadu	stem borer tolerance
11	Sirunsamba	Coimbatore, Tamil Nadu	stem borer and BPH tolerance
12	Ponkabi Samba	Tanjore, Tamil Nadu	blast tolerance
13	Lanumra	Assam	leaf folder and BLB tolerance
14	Yerra	Chittor, Andhra Pradesh	blast tolerance
15	Sampalpur	Central Province, India	leaf folder tolerance
16	Thekkan	Malabar, Kerala	stem borer and leaf folder tolerance
17	Natsama	Burma	better grain quality
18	Modan	Malabar, Kerala	blast tolerance
19	Cherumani	Malabar, Kerala	tolerance to sheath blight
20	Pattambi5	Pattambi, Kerala	blast tolerance
21	Pattambi8	Pattambi, Kerala	green leaf hopper (GLH) tolerance
22	Pattambi14	Pattambi, Kerala	tolerance to sheath blight
23	Pattambi20	Pattambi, Kerala	tolerance to sheath blight and gall fly
24	Pattambi32	Pattambi, Kerala	tolerance to sheath blight and blast
25	Pattambi37	Pattambi, Kerala	high yield potential
26	Pattambi51	Pattambi, Kerala	high yield potential
27	CO45	Coimbatore	high yield potential
28	CO46	Coimbatore	high yield potential
29	*Oryza rufipogon*	wild (AA)*	source of cytoplasmic male sterility
30	*O. punctata*	wild (CC)	multiple pest resistance
31	*O. Minuta*	wild (BBCC)	BPH, GLH, white-backed plant hopper (WBPH), BLB and blast resistance
32	*O. officinalis*	wild (BBCC)	BPH, GLH and WBPH resistance
33	*O. latifolia*	wild (CCDD)	high biomass production
34	*O. grandiglumis*	wild (CCDD)	high biomass production
35	*O. australeinsis*	wild (EE)	drought tolerance and BPH tolerance

*The letters in parentheses indicate the genomic status of the species

ing temperature was adjusted basing on the specific requirements of each primer combination. The PCR products were electrophoresed in 3% agarose gels at 100 V for 2 h. The gels were next stained in ethidium bromide for 30 min, de-stained for 15–30 min, and then observed under a UV transilluminator.

Data analysis

Each SSR band was scored as present (1), absent (0), or as a missing observation for each genotype. An accession was assigned a null allele for a microsatellite locus whenever an amplification product could not be detected for a particular genotype-marker combination. To measure the informativeness of the markers, the polymorphism information content (PIC) for each SSR locus was calculated according to the formula (Weir 1996): $PIC = 1 - (\Sigma p_i^2)$, where i is the total number of alleles detected for a SSR marker, and p_i is the frequency of the i^{th} plus allele in the set of the 35 rice genotypes investigated. PIC is also an estimate of the discriminatory power of a SSR marker locus. The frequencies of null alleles were not included in the calculation of PIC values.

Genetic similarity (GS) between genotypes i and j was estimated by using Jaccard's coefficient, as described by Sneath and Sokal (1973). Markers with missing observations for genotype i and/or j

Figure 1. Map showing the distribution of rice genotypes used in the present study across the Indian subcontinent. (Asterisks indicate the collection sites of genotypes as indicated in Table 1.)

package WINBOOT, developed at the International Rice Research Institute (Yap and Nelson 1996).

Results

Polymorphism of SSR markers

A total of 25 microsatellite markers dispersed across the genome were used to assess the extent of genetic diversity across the 35 rice genotypes. All 25 SSR primers generated polymorphic patterns. The distribution of alleles for a single SSR locus, *viz.* RM 229, across 28 rice landraces/cultivars, is shown in Figure 2 and across the 7 wild relatives in Figure 3. A total of 102 alleles were detected by the 25 markers. The number of alleles per locus varied from 3 to 8, with a mean of 4.86 alleles per locus (Table 2). The markers

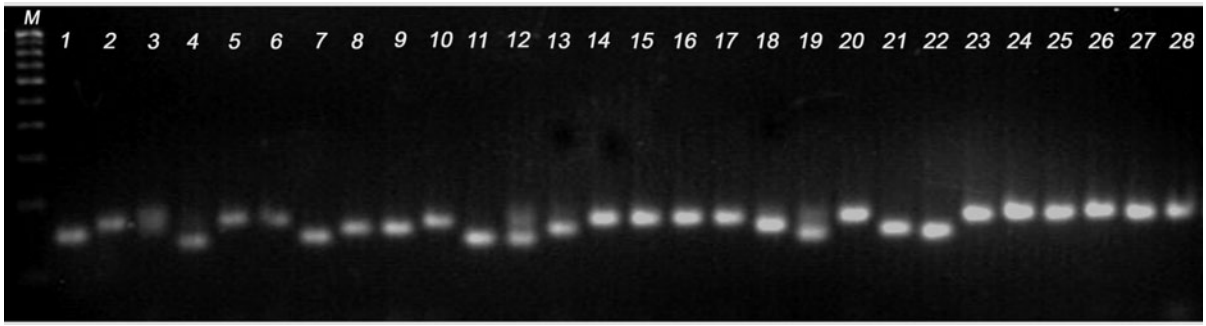

Figure 2. PCR products of the microsatellite marker RM 229 for 28 rice land races and cultivars. Lanes 1–19 = landraces; 20–28 = cultivars as in Table 1; M = DNA ladder.

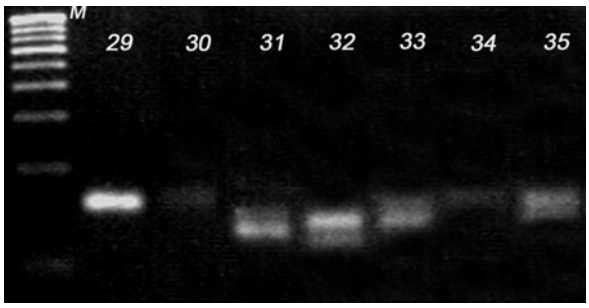

Figure 3. PCR products of the microsatellite marker RM 229 for 7 wild rice relatives. Lanes 29–35 as in Table 1; M = DNA ladder.

were not included in the calculation of GS_{ij}. Basing on the genetic similarity matrix, an unweighted pair group method of arithmetic averages (UPGMA) cluster analysis was used to assess the pattern of diversity among the rice genotypes. All calculations were performed by using NTSYS-pc version 2.1 software (Rohlf 2000). The robustness of the clusters in the dendrogram was tested by bootstrapping with the software

showed a high level of polymorphism, ranging from 66.7% (RM 136) to 100% (as many as 20 markers). The PIC values, derived from allelic diversity and frequency among the genotypes, were not uniform for all of the SSR loci tested. The PIC value for 25 primers varied from 0.498 (RM 265) to 0.89 (RM 206), with a mean of 0.707. In the set of 35 accessions, 28 loci showed null alleles. The frequency of accessions revealing null alleles varied from a minimum of 1 (for primers RM 130, RM 216 and RM 138) to a maximum of 13 for primer RM 224.

Genetic diversity levels

The Jaccard's genetic similarity (GS) values among rice landraces varied from 0.13 (between Lanumra from Assam and Ponkambi Samba from Tamil Nadu) to 0.90 (between the landraces Sadaisamba and Kalarsamba, both from Tamil Nadu). GS values among cultivars varied from

Table 2. Characteristics of the SSR markers used and their chromosome location, product size, number of polymorphic alleles, and PIC values calculated for a set of 35 rice genotypes

SSR marker	Sequence	Chro-mo-some loca-tion	Product size (bp)	Total no. of alleles	No. of poly-mor-phic alleles	% Polymo-rphism	PIC
RM 130	F: TGTTGCTTGCCCTCACGCGAAG R:GGTCGCGTGCTTGGTTTGGTTC	3	85	3	3	100.00	0.548
RM 206	F: CCCATGCGTTTAACTATTCT R: CGTTCCATCGATCCGTATGG	11	147	8	8	100.00	0.891
RM 224	F: ATCGATCGATCTTCACGAGG R: TGCTATAAAAGGCATTCGGG	11	157	8	8	100.00	0.724
RM 216	F: GCATGGCCGATGGTAAAG R: TGTATAAAACCACACGGCCA	10	146	4	3	75.00	0.624
RM 229	F: CACTCACACGAACGACTGAC R: CGCAGGTTCTTGTGAAATGT	11	116	6	6	100.00	0.814
RM 235	F: AGAAGCTAGGGCTAACGAAC R: TCACCTGGTCAGCCTCTTTC	12	124	7	7	100.00	0.763
RM 257	F: CAGTTCCGAGCAAGAGTACTC R: GGATCGGACGTGGCATATG	9	147	3	3	100.00	0.521
RM 265	F: CGAGTTCGTCCAAGTGAGC R: CATCCACCATTCCACCAATC	1	106	5	4	80.00	0.498
RM 272	F: AATTGGTAGAGAGGGGAGAG R: ACATGCCATTAGAGTCAGGC	1	119	3	3	100.00	0.641
RM 240	F: CCTTAATGGGTAGTGTGCAC R: TGTAACCATTCCTTCCATCC	2	132	4	4	100.00	0.832
RM 138	F: AGCGCAACAACCAATCCATCCG R: AAGAAGCTGCCTTTGACGCTATGG	2	233	3	2	66.67	0.546
RM 208	F: TCTGCAAGCCTTGTCTGATG R: TAAGTCGATCATTGTGTGGACC	2	173	4	4	100.00	0.745
RM 159	F: GGGGCACTGGCAAGGGTGAAGG R: CTTGTGCTTCTCTCTCTCTCTCTCTCTC	5	248	5	5	100.00	0.689
RM 254	F: AGCCCCGAATAAATCCACCT R: CTGGAGGAGCATTTGGTAGC	11	165	6	6	100.00	0.721
RM 270	F: GGCCGTTGGTTCTAAAATC R: TGCGCAGTATCATCGGCGAG	12	108	4	3	75.00	0.664
RM 220	F: GGAAGGTAACTGTTTCCAAC R: GAAATGCTTCCCACATGTCT	1	127	5	5	100.00	0.841
RM 236	F: GCGCTGGTGGAAAATGAG R: GGCATCCCTCTTTGATTCCTC	2	191	4	4	100.00	0.756
RM 248	F: TCCTTGTGAAATCTGGTCCC R: GTAGCCTAGCATGGTGCATG	7	102	5	5	100.00	0.828
RM 421	F: AGCTCAGGTGAAACATCCAC R: ATCCAGAATCCATTGACCCC	5	234	6	6	100.00	0.715
RM 148	F: ATACAACATTAGGGATGAGGCTGG R: TCCTTAAAGGTGGTGCAATGCGAG	3	129	4	4	100.00	0.812
RM 226	F: AGCTAAGGTCTGGGAGAAACC R: AAGTAGGATGGGGCACAAGCTC	1	274	5	5	100.00	0.849
RM 184	F: ATCCCATTCGCCAAAACCGGCC R: TGACACTTGGAGAGCGGTGTGG	10	219	4	4	100.00	0.654
RM 204	F: GTGACTGACTTGGTCATAGGG R: GCTAGCCATGCTCTCGTACC	6	169	6	5	83.33	0.551
RM 338	F: CACAGGAGCAGGAGAAGAGC R: GGCAAACCGATCACTCAGTC	3	183	6	6	100.00	0.742
RM 114	F: CAGGGACGAATCGTCGCCGGAG R: TTGGCCCCCTTGAGGTTGTCGG	3	209	7	7	100.00	0.664
	Mean			4.86	4.67	95.2	0.707

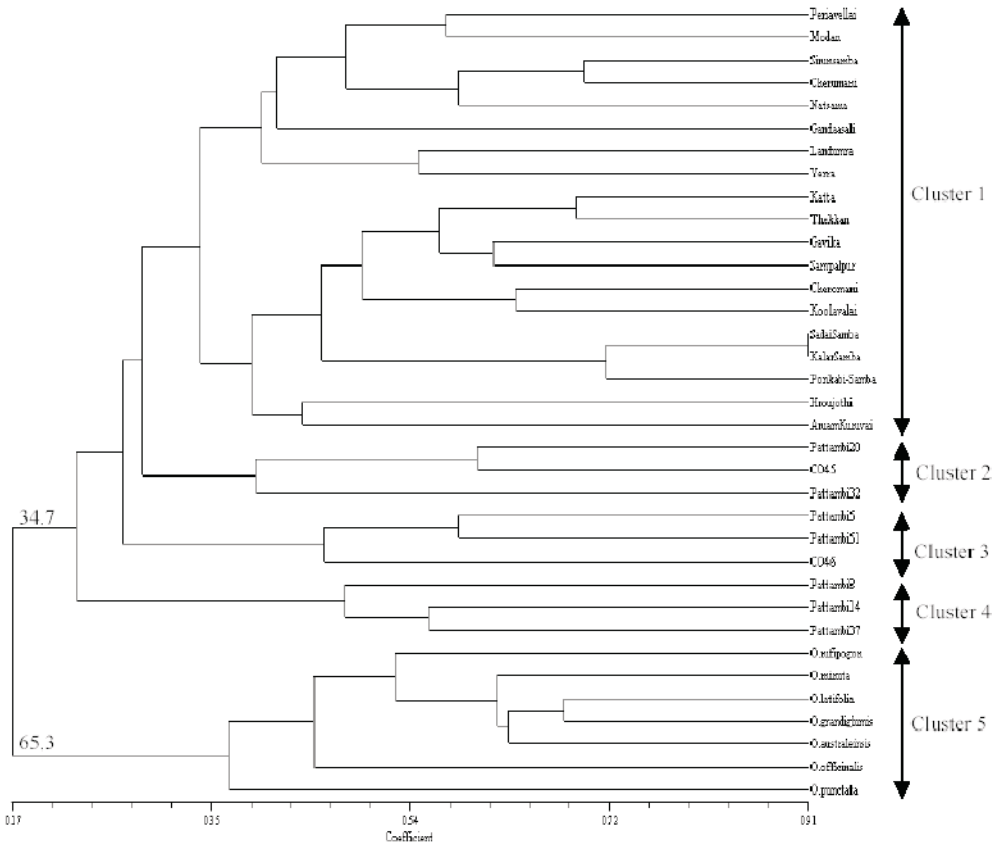

Figure 4. Dendrogram resulting from UPGMA cluster analysis of 35 rice genotypes, based on data derived from 25 microsatellite markers. The values at the forks show the percentage contribution of the group bracketed by the fork by bootstrapping.

0.125 (between Pattambi 8 and Pattambi 51) to 0.583 (between Pattambi 51 and Pattambi 5). In the case of wild relatives, genetic similarity values varied from 0.2 (between *O. punctata* and *O. officinalis*) to 0.441 (between *O. grandiglumis* and *O. latifolia*).

Genetic diversity pattern

The cluster dendrogram (Figure 4) revealed 5 clusters that were demarcated at a cut-off similarity coefficient level of 0.30, below which the similarity values narrowed conspicuously. Distinct clusters could be observed among landraces, cultivars and wild relatives. Cluster I was the largest and included all 19 landraces, while clusters II, III and IV were of equal size, comprising 3 cultivars each. Cluster V included all 7 wild relatives. The cluster-wise allelic diversity was computed to compare diversity within various clusters (Table 3). Allelic diversity varied from 0.175 (cluster IV) to 0.225 (cluster III) for the cultivar groups. Cluster I, which included only landraces, exhibited an allelic diversity of 0.356. The highest value of allelic diversity (0.436) was recorded in cluster V, which comprised solely the wild relatives.

Table 3. Composition and size of clusters along with allelic diversity values among rice accessions

Cluster number	No. of genotypes	Nature of genotypes	Allelic diversity
I	19	landraces	0.356
II	3	cultivars	0.200
III	3	cultivars	0.225
IV	3	cultivars	0.175
V	7	wild	0.436

Discussion

The characterization and quantification of genetic diversity within closely related crop germplasm has long been a major goal, as it is essential for a rational use of genetic resources. Above and beyond, the analysis of genetic variation among

breeding materials is of fundamental interest to plant breeders, as it contributes immensely to selection, monitoring of germplasm, and also to prediction of potential genetic gains (Chakravarthy and Rambabu 2006).

Diversity based on phenological and morphological characters varies between environments, and its evaluation requires growing plants to full maturity. Markers based on expressed gene products, proteins or isozymes, are also influenced by the environment and reveal a low level of polymorphism and low abundance (Ravi et al. 2003). In contrast, DNA-based 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, characterized by abundance and untouched by environmental influence (Powell et al. 1996). Xio et al. (1996) demonstrated the remarkable potential of microsatellite markers to discriminate between rice genotypes, as compared to other molecular markers. Ravi et al. (2003) could also generate unique SSR profiles in rice by using a few primers that covered all 12 chromosomes.

In the present investigation, SSR marker loci generated by 25 primer pairs were used to assess the genetic diversity among 35 rice genotypes. The SSR primers generated 102 alleles with the number of alleles per locus varying from 3 to 8. The average number of alleles per locus was 4.86, indicating a greater magnitude of diversity among the plant materials included in this investigation. The study also revealed that primers RM 206 and RM 224 on chromosome 11 generated a maximum of 8 bands, while primer RM 138 in chromosome 2 had a minimum of 2 bands. Many studies have also reported significant differences in allelic diversity among various microsatellite loci (Akagi et al. 1997; McCouch et al. 2001; Ravi et al. 2003). The alleles revealed by markers showed a high degree of polymorphism, with as many as 20 producing 100% of bands polymorphic. This amply suggested that the genotypes selected for this study harboured enough genetic divergence. The markers showed an average PIC value of 0.705, which confirms that SSR markers used in this study were highly informative, because PIC values higher than 0.5 indicate high polymorphism. Markers with PIC values of 0.5 or higher are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate of a marker at a specific locus (DeWoody et al. 1995).

The mean PIC value observed in this study was higher than the PIC value of 0.578 recorded by Ravi et al. (2003) in an earlier study among rice cultivars, landraces and wild relatives. This could indicate that the genotypes used in the present study were more diverse due to differences in origin, ecotype and speciation. Microsatellite markers exhibit high PIC values because of their codominant expression and multiallelism (Ferreira and Grattapaglia 1998). Nevertheless, 25 microsatellite markers were able to discriminate between the landraces /cultivars/wild relatives and demonstrated a maximum genetic similarity value of 0.91 between the landraces Sadaisamba and Kalarsamba. These landraces both originated from Tamil Nadu and had similar morphological features, such as a taller and erect plant type, short bold and awnless grains. The minimum genetic similarity value of 0.125 was observed between the landraces Lanumra and Ponkambi Samba, consistent with their diverse origins from Assam and Tamil Nadu, respectively. Panaud et al. (1996) and Chakravarthy and Rambabu (2006) in their studies using SSR markers in rice, described similarly high genetic similarity among landraces of common geographic origin and low similarity among landraces of diverse geographic origins. Among wild relatives, a maximum similarity value of 0.682 was observed between *O. grandiglumis* and *O. latifolia*, which was consistent with their common genomic status (CCDD). Among the wild species, the minimum similarity value (0.200) was observed among the wild species *O. punctata* and *O. officinalis*, which was also consistent with their differential ploidy status, *viz*. diploid (CC) and tetraploid (BBCC), respectively. The efficient use of SSR markers to discriminate between *Oryza* species with various genomes was also demonstrated by Bautista et al. (2006) and Cai and Morishima (2002). The multiallelic nature of SSR markers has the unambiguous advantage of discriminating between the genotypes more precisely. UPGMA cluster analysis of the SSR-based genetic similarity matrix resulted in the classification of cultivars, landraces and wild relatives into separate clusters. Moreover, varietal profiling based on SSR markers will be more reliable as compared to profiling based on other markers, since SSR markers detect finer levels of variations among closely related lines. The dendrogram (Figure 4) resulting from UPGMA analysis could reveal allelic richness of 5 clusters for various sizes at a similarity coefficient level of 0.30. Cluster I

was the largest and included all the 19 landraces, with a mean allelic diversity of 0.356, while clusters II, III and IV included 3 cultivars each, with allelic diversity values of 0.200, 0.225 and 0.175, respectively. The allelic diversity for cluster V was the highest (0.436), as this cluster included only the wild relatives. The bootstrap values for the 2 major clades that separate the cultivars-landraces and wild species were substantially lower than the 95% confidence rule of bootstrapping (Felsenstein 1985). The very low bootstrap value (34.7%) observed in the clade that comprised cultivars and landraces indicate that the relative position of each accession may vary within the cluster if the dendrogram is rebuilt. A comparison of values of allelic diversity among the cultivars, as compared to landraces and wild relatives, clearly emphasize the scope for introgression of genes through hybridization of landraces and wild relatives with the cultivars for increasing genetic diversity in the cultivated rice gene pool (Vaughan 1991; Ishikawa et al. 2006). This also reiterates the need for genetic diversity evaluation among the principal genotype classes and cataloguing them for the benefit of the future.

In conclusion, the allelic diversity revealed by 25 SSR primers was sufficient enough to distinguish between the cultivars, landraces and wild relatives. The allelic variation was lower within the cultivar group than among landraces and wild germplasm, indicating the possibility to exploit distant relatives to broaden the genetic base of rice.

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