RESEARCH ARTICLE

Molecular evaluation of genetic diversity and association studies in rice (*Oryza sativa* L.)

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Abstract

In the present study, we tested rice genotypes that included un(der)exploited landraces of Tamil Nadu along with *indica* and *japonica* test cultivars to ascertain their genetic diversity structure. Highly polymorphic microsatellite markers were used for generating marker segregation data. A novel measure, allele discrimination index, was used to determine subpopulation differentiation power of each marker. Phenotypic data were collected for yield and component traits. Pattern of molecular differentiation separated *indica* and *japonica* genotypes; *indica* genotypes had two subpopulations within. Landraces were found to have *indica* genome, but formed a separate subgroup with low linkage disequilibrium. The landraces further separated into distinct group in both hierarchical clustering analysis using neighbour-joining method as well as in the model based population structure analysis. *Japonica* and the remaining *indica* cultivars formed two other distinct groups. Linkage disequilibrium observed in the whole population was considerably reduced in subpopulations. Low linkage disequilibrium of landforms suggests their narrow adaptation in local geographical niche. Many population specific alleles could be identified particularly for *japonica* cultivars and landraces. Association analysis revealed nine marker–trait associations with three agronomic traits, of which 67% were previously reported. Although the testing landraces together with known cultivars had permitted genome-wide association mapping, the experiment offers scope to study more landraces collected from the entire geographical region for drawing more reliable information.

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Introduction

Rice (*Oryza sativa* L.) is staple food for more than half of the world population and is one of the most important food crops grown worldwide (Sasaki and Burr 2000). Few of the large *ex situ* germplasm collections in world belongs to this crop (Jackson and Juggan 1993). Although there are more than 40,000 rice varieties reported worldwide, a small fraction of these have been used in practical breeding. Therefore, better understanding of the genetic makeup of underused rice germplasm is an important issue for rice breeding. Recent advent of molecular and computational tools now enables the estimation of genetic diversity and population structure of rice germplasm rather easily.

During the agricultural modernization era, a large number of landforms of rice have been lost, when modern high-yielding cultivars replaced them. This was a requirement at that period, because in the absence of organized breeding, low-yielding landraces could not produce enough food grains to tide over natural calamities and famines. It is well known that during early domestication and evolutionary selection, cultivated rice differentiated into two major subspecies, *indica* and *japonica* (Chang 1976; Oka 1988; Morishima *et al.* 1992). Although significant differences exist in morphological and physiological characters between the two subspecies (Liu 1993), classifying them based on these traits is seldom unambiguous. Further, early landforms can be carrying cross-subspecies genetic variations conserved within them. In this context, molecular evaluation, particularly of genetic diversity can be useful.

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In tropical Asian countries such as India, few of the traditional native landraces are still under cultivation by resource poor farmers who practice subsistence farming (Ram *et al.* 2007). Although less productive, these landraces have shown excellent adaptation to local conditions and they are known to harbour great genetic potential for rice improvement, particularly for stress tolerance (Hanamaratti *et al.* 2008; Lisa *et al.* 2011) and quality (Huang *et al.* 2010; Pervaiz *et al.* 2011). Therefore, the need to characterize available landraces has become important in modern day crop improvement (Dale *et al.* 1985; Rezai and Frey 1990). In an unconventional approach, we attempted to study a small set of un(der)exploited landraces, by evaluating them along with well-established *indica* and *japonica* cultivars, using the methods for analysis of population structure. The objectives of this study were to (i) decipher genetic diversity structure of the landraces in comparison with both *indica* and *japonica* cultivars using molecular markers; (ii) to assess the linkage disequilibrium (LD) structure within the landraces; and (iii) to assess the utility of association mapping approaches in mapping marker–trait associations in the population.

Materials and methods

Plant materials

Rice landraces randomly collected from Tamil Nadu, southern India, maintained in the germplasm collection at Tamil Nadu Agricultural University, Coimbatore, India, were used in this study. These lines are known to possess stress resistance to abiotic factors especially for drought (Thiyagarajan and Selvaraju 2001; Gomez and Kalamani 2002; Anbumalarmathi *et al.* 2008; Muthuramu *et al.* 2011). In order to decipher the evolutionary relationship of 10 selected landraces, they were evaluated along with *indica* and *japonica* genotypes (table 1). One entry among landraces, Norungan was an established drought tolerant variety (Subashri *et al.* 2009). *Indica* accessions comprised of 21 independently derived varieties (IDV) and *japonica* accessions included five temperate and four tropical *japonica* cultivars. Since the abiotic factors of drought and salt stress have overlapping tolerance mechanisms, among the IDV drought-tolerant, salt-tolerant and high-yielding varieties were included for effective characterization. Specific phenotyping for tolerance, however, was not attempted in this study.

Genotyping and phenotyping

Genotypes were sown in rock wool and grown in controlled greenhouse conditions at Plant Research International, Wageningen, The Netherlands, for genotyping and were later field evaluated at Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India. Young leaves were harvested after 21 days of sowing in a deep well block, containing one tungsten carbide bead (3 mm) in each well, frozen in liquid nitrogen, lyophilized and ground in a Retch shaking mill prior to DNA extraction. DNA extraction was performed according to Fulton *et al.* (1995). A panel of 96 highly polymorphic and diverse rice microsatellite markers distributed across 12 chromosomes were selected from 300 markers initially used for the polymorphism survey. The PCR amplicons were resolved by MetaPhor® (FMC BioProducts, Rockland, USA) agarose gels stained with ethidium bromide, and visualized under UV transillumination. The amplicon data were scored based on the product size, as revealed in comparison with the standard 1 kb DNA ladder used in the resolving gel.

For the field study, varieties were grown in randomized complete block design with three replications, adopting a spacing of 20 cm between rows and 15 cm between plants with standard agronomic management. Phenotype data were recorded on plant height, panicle length, number of filled grains per panicle, weight of hundred grains and single plant yield from 10 individual plants per genotype and were averaged.

Diversity and population structure

The SSR allele segregation data were used to construct dissimilarity matrix between genotypes using simple matching coefficient (Sokal and Michener 1958). The dissimilarity matrix was used for clustering of genotypes, based on unweighted neighbour-joining method (NJ). Analysis was performed using DARWin 5.0 (Perrier *et al.* 2003). To check the goodness of fit of the clustering, a cophenetic correlation was computed between similarity and the cophenetic matrices (Rohlf and Sokal 1981). Confidence limits of different clades were tested by bootstrapping 10,000 times to assess the repetitiveness of genotype clustering (Felsenstein 1985).

For SSR markers, polymorphism information content (PIC), was calculated as the measure of informativeness of markers (Botstein *et al.* 1980). As an alternative to graphical clustering methods, a model-based Bayesian approach implemented in the software package Structure 2.3.3 (Pritchard *et al.* 2010) was used to analyse the population structure of rice accessions. Optimum number of populations was inferred by running an admixture ancestry model with correlated allele frequencies starting from two populations $K = 1$ to $K = 10$, with three runs at each K. For each run, 500,000 burn-ins followed by 500,000 Markov chain Monte Carlo (MCMC) simulations were performed. The ideal value of K was determined from the uppermost hierarchical level of population structure, detected using an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values (Evanno *et al.* 2005). In addition, the ideal number according to Pritchard *et al.* (2000) was used as the criterion for defining the number of groups (k). The most trustworthy value was estimated based on the lowest negative number of Ln (the log-likelihood of the data) and the lowest standard deviation found during statistical analysis. Inferred ancestry estimates of individuals

Table 1. Landraces and test varieties, and their characteristic attributes.

Genotype	Subtype*	Attributes	Reference
Kallurundaikar	*indica*	Landrace, drought-tolerant	Anbumalarmathi *et al.* (2008)
Chandaikar	*indica*	Landrace, drought-tolerant	Anbumalarmathi *et al.* (2008)
Vellachitraikar	*indica*	Landrace, drought-tolerant	Anbumalarmathi *et al.* (2008)
Nootripathu	*indica*	Landrace, drought-tolerant	Muthuramu *et al.* (2011)
Mattaikar	*indica*	Landrace, drought-tolerant	Muthuramu *et al.* (2011)
Norungan	*indica*	Landrace, drought-tolerant	Muthuramu *et al.* (2011)
Sivappuchitraikar	*indica*	Landrace, drought-tolerant	Gomez and Kalamani (2002)
Poongar	*indica*	Landrace, drought-tolerant	Gomez and Kalamani (2002)
Kuliyadichan	*indica*	Landrace, drought-tolerant	Gomez and Kalamani (2002)
Varappukudanchan	*indica*	Landrace, drought-tolerant	Gomez and Kalamani (2002)
PMK 2	*indica*	Variety, drought-tolerant, upland	Vikas *et al.* (2009)
ASD 16	*indica*	Variety, high quality	Manoharan *et al.* (1993)
ASD 20	*indica*	Variety, high yield	Shanmugasundaram *et al.* (1997)
ADT 43	*indica*	Variety, high yield	Geetha *et al.* (2006)
IR 64	*indica*	Variety, good quality	Khush and Virk (2005)
IR 66	*indica*	Variety, high yield	Khush and Virk (2005)
TRY 1	*indica*	Variety, salt-tolerant, high yield	Rajagoplan *et al.* (2004)
TRY 2	*indica*	Variety, salt-tolerant, high yield	Rajagoplan *et al.* (2004)
SAT1806	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1807	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1808	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1810	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1812	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1813	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1815	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1816	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1817	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1818	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1820	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1824	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
SAT1827	*indica*	Variety, salt-tolerant	Mahalingam *et al.* (2004)
Jing-Xi 17	Tempjap	Cold injury tolerant	Qian *et al.* (2000)
Moroberekan	Tropjap	Drought, blast-tolerant	McCough and Doerge (2005)
Nipponbare	Tempjap	Round grain, high yielding	Ishimaru (2003)
Akihikari	Tempjap	high yielding, good quality	Ishikawa *et al.* (1988)
Koshihikari	Tempjap	Short grains, good quality	Ishikawa *et al.* (1988)
Azucena	Tropjap	Upland rice, drought-tolerant	Price *et al.* (2002)
Taichung 65	Tropjap	Widely adapted, high yield	Tsai and Oka (1965)
Sinampaga Sel.	Tropjap	Long grain	Lu *et al.* (2005)
Lemont	Tropjap	High yield, high milling, semidwarf	Bollich *et al.* (1985)

* Tempjap, temperate *japonica*; tropjap, tropical *japonica.*

(Q-matrix) were derived for the selected subpopulation (Pritchard *et al.* 2000).

Since the genotypes contained two subspecies, the power of each SSR marker (m) in differentiating the two major subpopulations of *indica* (i) and *japonica* (j) was estimated by taking the average of absolute difference of the frequencies of the positive alleles for both subtypes. We call this as the allele discrimination index (D_m),

$$D_m(ij) = \frac{1}{k}\sum_{i=1}^{k} |\hat{p}_a^i - \hat{p}_a^j|$$

where, $\hat{p}_a^i$ and $\hat{p}_a^j$ are the frequencies of positive allele a for subpopulations i and j, respectively.

Linkage disequilibrium

LD was estimated for each pair of SSR loci using Tassel 2.1 software (Bradbury *et al.* 2007), both in overall population and in subpopulations. LD was measured using D' and r^2 estimates modified for multiple loci (Hedrick 1987). Significance (P values) of D' for each SSR pair was determined by 100,000 permutations.

Phenotype diversity and LD mapping

Agronomic data were analysed for phenotypic diversity using principal component approach using simple correlations. Principal component scores obtained for each genotype were used for computing squared Euclidean distances, and

Table 2. Chromosome wise marker allele segregation statistics showing polymorphic information content and allele discrimination index (D_m) and number of population specific alleles.

	SSR marker types			Allele number				Number of population specific alleles*		
Chromosome	Bi	Tri	Tetra	Total	Mean	PIC	D_m	*POP1*	*POP2*	*POP3*
1	4	2	1	18	2.57	0.35	0.49	1	2	1
2	7	5	1	33	2.54	0.35	0.66	8	–	1
3	4	3	1	21	2.63	0.4	0.37	3	2	–
4	5	2	1	20	2.5	0.25	0.37	3	3	1
5	3	5	1	25	2.78	0.38	0.57	7	–	2
6	5	3	–	22	2.75	0.32	0.56	3	2	1
7	3	2	1	16	2.67	0.34	0.61	2	2	1
8	8	-	–	16	2.00	0.3	0.69	2	–	–
9	5	1	–	13	2.17	0.31	0.6	5	–	–
10	7	2	–	20	2.22	0.28	0.57	2	1	–
11	5	2	1	20	2.50	0.33	0.55	4	1	–
12	5	1	–	13	2.17	0.31	0.66	3	–	–
Total	5.08	2.55	1.00	237	2.47	0.33	0.6	43	13	7

**POP1*, *POP2* and *POP3* are the estimated subpopulations.

grouping of genotypes were performed using unweighted NJ method (Gascuel 1997) with bootstrapping of 10,000 iterations. LD mapping (association mapping) was performed to analyse marker–trait association by structured association approach using ancestry coefficient (Q values) estimates as covariate in a general linear model (GLM) function using TASSEL 2.1. For each marker–trait combination, GLM estimated the ordinary least squares solution (Searle 1987). Multiple testing corrections were performed by adjusting maker probability values for multiple test runs, by a permutation test derived using a step-down MinP procedure (Ge *et al.* 2003). The significant association for a marker and trait was selected when adjusted *P* value (false discovery rate) was below 0.05.

Results

SSR marker segregation

Ninety-six SSR markers were generated from 237 alleles with an average of 2.5 alleles per marker (table 2). The number of alleles per marker varied from two to four. The overall frequency of biallelic markers was high (63.5%), followed by triallelic (26.1%) and tetraallelic (10.4%). The PIC values ranged from 0.09 (*RM144*) to 0.60 (*RM21*) (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Comparison of SSR polymorphism based on markers anchored to different linkage groups (table 2) showed that average PIC values ranged from 0.25 (chromosome 4) to 0.40 (chromosome 3). Chromosome 9 had maximum average allele discrimination index of 0.84 followed by chromosome 2 (0.66), while that of chromosome 10 was the lowest (0.35). Nine markers (*RM29*, *RM318*, *RM48*, *RM349*, *RM182*, *RM38*, *RM201*, *RM316* and *RM235*) distributed over chromosomes 2, 4, 7, 8, 9 and 12 were

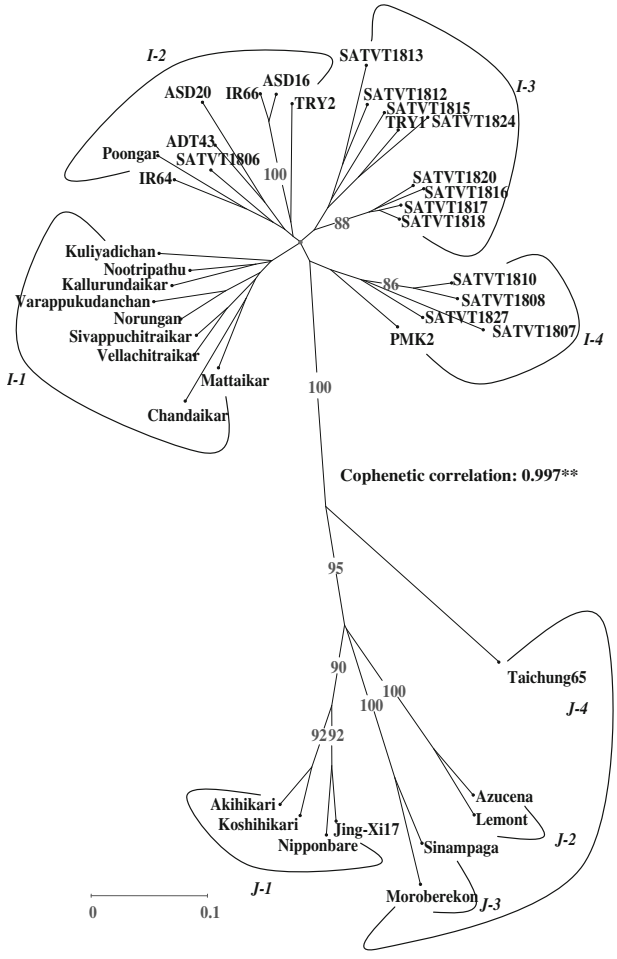

Figure 1. Dendrogram of 40 rice genotypes by unweighted neighbour-joining of simple matching coefficients based on SSR segregation data.

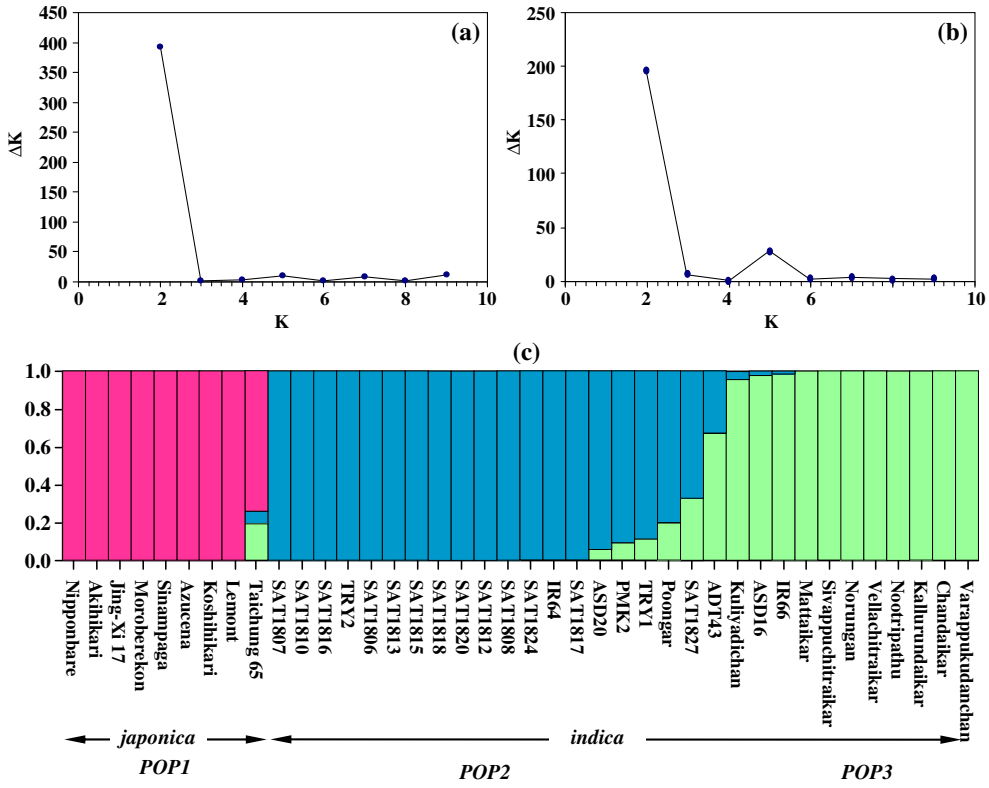

Figure 2. Analysis of population structure showing (a) values of ΔK for determining optimum number of subpopulations for total population (b) ΔK for *indica* accessions and (c) bar plot showing distribution of genotypes within subpopulations.

found to have the maximum allele discrimination index (D_m) value of 1.0. They differentiated *indica* and *japonica* genotypes absolutely. The lowest D_m was recorded for the marker *RM216* (0.02), besides 10 other markers showing less than 10% discrimination power (*RM7*, *RM280*, *RM169*, *RM18*, *RM264*, *RM216*, *RM228* and *RM144*).

Genotypic diversity

The dendrogram constructed from the matrix of simple matching coefficients revealed two major clusters (figure 1), separating genotypes of *indica* and *japonica*. The bootstrap confidence level was 100% for each of these clades. Besides a highly significant cophenetic correlation value of 0.99 was obtained on comparison of distance and cophenetic matrices. The genotypes of *indica* group was further grouped into four subclusters, named from *I*-1 to *I*-4. All but one of the tolerant landraces, Poongar, were found grouped into cluster *I*-1. 'Poongar' was clustered together with high-yielding cultivars in *I*-2. The cluster *I*-3 contained most of the salt-tolerant genotypes, while the remaining genotypes were found grouped into cluster *I*-4. The *japonica* genotypes were differentiated into four clusters. The first subcluster (*J*-1) consisted of four genotypes, Nipponbare, Jing-Xi17, Akihikari and Koshhikari, all temperate *japonica* genotypes with a bootstrap coverage probability of >90%. Of the remaining, tropical *japonica* genotypes were separated into two subgroups, Lemont and Azucena falling in cluster *J*-2, and Moroberekan and

Table 3. Population statistics of the estimated subpopulations.

Populations*	Membership (%)	F_{ST}	Expected heterozygosity	Allele frequency divergence		
				POP1	*POP2*	*POP3*
POP1	21.9	0.530	0.197	–	–	–
POP2	44.3	0.613	0.178	0.453	–	–
POP3	33.8	0.641	0.258	0.473	0.058	–

**POP1*, *POP2* and *POP3* are the estimated subpopulations.

Sinampaga selection into *J*-3, while Taichung65 was falling into cluster *J*-4.

Population structure

Analysis of population structure distinguished subspecific populations of *indica* and *japonica* (figure 2a), with a highest ΔK value of 391.7. All *japonica* cultivars were grouped into one population (*POP1*) with a membership proportion of 21.9% (table 3). Since the landraces were found to share very little allelic relationship with *japonica* cultivars, a further analysis of subpopulation structure within *indica* genotypes was attempted to reveal two subpopulations as per the maximum ΔK of 214.2 (figure 2b). Among the subpopulations, *POP3* with a membership proportion of 33.8% consisted of nine landraces except one, Poongar. *POP2* contained *indica* cultivars, and the landrace Poongar. The bar plot (figure 2c) shows the distribution of genotypes within and between subpopulations based on the inferred ancestry coefficients (see table 2 in electronic supplementary material). The fixation index (F_{ST}) values of subpopulations ranged from 0.53 (*POP1*) to 0.64 (*POP3*), while the pairwise allele frequency divergence values were maximum between *indica* and *japonica* subpopulations. Allele frequency divergence between *indica* subpopulations, *POP2* and *POP3* was 0.058.

There were 43 alleles that differentiated *japonica* accessions (*POP1*) from rest of the population, of which eight were from chromosome 2 followed by seven from chromosome 5. Subspecies-specific private alleles were low (20) for differentiating *indica* genotypes, of which seven were specific to population of landraces (table 2). Seventeen population-specific alleles distributed on eight chromosomes showed maximum allele discrimination index of 1.00 (see table 3 in electronic supplementary material) in *POP1*. The proportion of such markers was more in chromosome 2 (four alleles) followed by chromosome 9 (three alleles). Among *indica* accessions, no population specific allele showed maximum allele diversity of 1.0.

Linkage disequilibrium

Among the 4559 pairs of SSR loci, 2104 pairs (46.2%) showed significant ($P < 0.05$) LD for the total population (table 4). LD dropped drastically within deciphered subpopulations, with *POP2* having 5.6% LD, followed by 5% in *POP1* and 0.8% in *POP3*. The genotypes of the *indica* group altogether had 5.9% LD. Marker pairs in significant LD on same chromosome were greatly reduced among subpopulations, with *POP1* and *POP2* having seven pairs each in LD, and none occurred in *POP3*. These seven pairs were distributed on four chromosomes (2, 5, 6 and 11) in *POP1*, while in *POP2* they were located on chromosomes 1, 2, 4, 5 and 11. LD scatter plot showed reduction in LD as interval distances between marker pairs increased. There was reduction in number as well as in strength of LD as the distance between marker pairs increased (figure 3, a & b).

Phenotypic diversity

Phenotypic variation for five agronomic traits is given in table 5. Significant positive associations were observed between plant height and panicle length (0.54) and grain yield/plant and weight of 100 grains (0.43). Number of filled

Table 4. Proportion of SSR marker pairs in significant ($P < 0.05$) linkage disequilibrium.

	POP1		*POP2*		*POP3*		*Indica*		Total	
Marker pairs	No. pairs	*P*	No. pairs	*P*	No. pairs	*P*	No. pairs	*P*	No. pairs	*P*
Interchromosomal	113 (2234)	0.051	106 (1857)	0.057	6 (685)	0.009	142 (2362)	0.060	1925 (4203)	0.458
Intrachomosomal	7 (181)	0.039	7 (159)	0.044	0 (56)	0.000	10 (194)	0.052	179 (356)	0.503
chrom. 1	0 (10)	0.000	2 (10)	0.200	0 (6)	0.000	0 (15)	0.000	9 (21)	0.429
chrom. 2	2 (28)	0.071	1 (21)	0.048	0 (3)	0.000	2 (21)	0.095	63 (78)	0.808
chrom. 3	0 (28)	0.000	0 (21)	0.000	0 (10)	0.000	5 (21)	0.238	9 (28)	0.321
chrom. 4	0 (15)	0.000	1 (10)	0.100	0 (3)	0.000	1 (15)	0.067	5 (28)	0.179
chrom. 5	1 (28)	0.036	2 (15)	0.133	0 (15)	0.000	1 (20)	0.050	27 (36)	0.750
chrom. 6	3 (28)	0.107	0 (21)	0.000	0 (0)	0.000	1 (21)	0.048	8 (28)	0.286
chrom. 7	0 (6)	0.000	0 (6)	0.000	0 (3)	0.000	0 (10)	0.000	10 (15)	0.667
chrom. 8	0 (15)	0.000	0 (10)	0.000	0 (1)	0.000	0 (15)	0.000	6 (28)	0.214
chrom. 9	0 (3)	0.000	0 (1)	0.000	0 (0)	0.000	0 (1)	0.000	15 (15)	1.000
chrom. 10	0 (11)	0.000	0 (28)	0.000	0 (3)	0.000	0 (28)	0.000	6 (36)	0.167
chrom. 11	1 (3)	0.333	1 (15)	0.067	0 (6)	0.000	0 (21)	0.000	12 (28)	0.429
chrom. 12	0 (6)	0.000	0 (1)	0.000	0 (6)	0.000	0 (6)	0.000	9 (15)	0.600
Overall	120 (2415)	0.050	113 (2016)	0.056	6 (741)	0.008	152 (2556)	0.059	2104 (4559)	0.462

Figures in parenthesis show total number of marker pairs; *P*, proportion of marker pairs in LD; *POP1*, *POP2* and *POP3* are the estimated subpopulations.

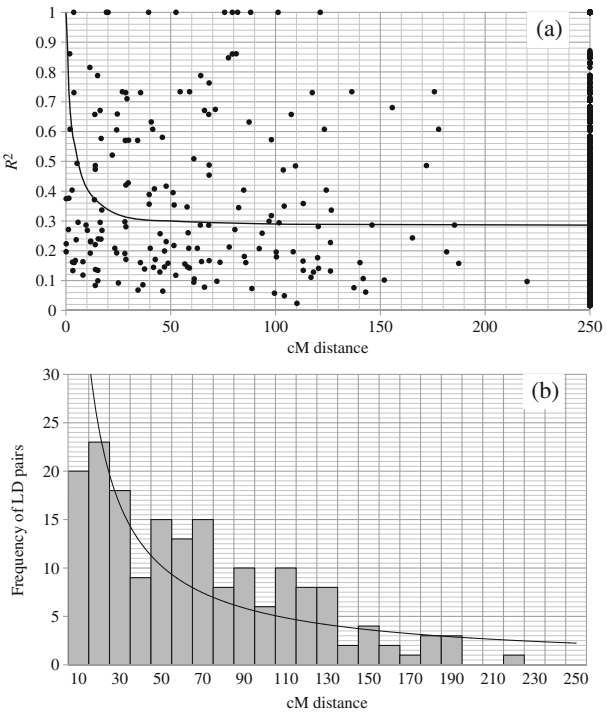

Figure 3. Intrachromosomal linkage disequilibrium scatter plot showing (a) R^2 values for marker pairs and (b) frequency of locus pair in LD plotted against marker intervals in cM. LD for unlinked (interchromosomal) pairs are plotted at 250 cM distance.

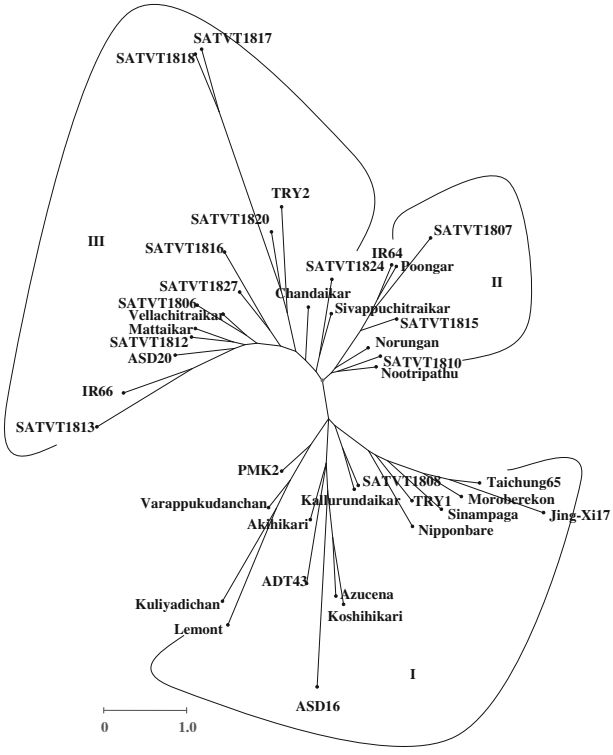

Figure 4. Dendrogram showing grouping of 40 genotypes based on agronomic traits.

grains per panicle and weight of 100 grains were negatively correlated (−0.32).

First three principal components derived from the correlation coefficients explained 86% of the total variation for the agronomic traits and had eigen values more than 1.0. The first component explained 38.5% of the variation, followed by 26.5% by the second and 21.6% by the third (table 5). Among the agronomic traits, plant height and panicle length showed higher contribution (34.3% and 24.1%, respectively) to first principal component, while grain yield/plant (50.7%) and numbers of grains/panicle (43.5%) showed significant contribution towards second principal component. Weight of 100 grains (46.2%) and panicle length (32.6%) showed higher contribution towards third component. Dendrogram constructed using principal component scores of genotypes, showed three distinct groups of genotypes (figure 4). The first group (group I) was dominated by the *japonica* genotypes, along with few of the landraces and elite cultivars such as TRY1, PMK2, ADT43 and ASD16. The second and third groups consisted entirely of *indica* genotypes.

Linkage disequilibrium mapping

GLM analysis of marker–trait association among the population revealed putative association of three markers (*RM302*,

Table 5. Phenotype statistics and principal component analysis.

Trait	Mean	Range	CV (%)	h^2 (%)	GA	Contribution to PC (%) PC1	PC2	PC3	Simple correlations			
SPY	11.35	4.8–18.5	24.63	56.3	7.37	12.2	50.7	0.3	SPY			
PNL	24.70	14.5–32.8	14.72	62.4	5.85	24.1	0.6	32.6	0.22	PNL		
PH	115.86	78.0–155.4	17.44	69.1	36.2	34.3	2.0	7.2	0.22	0.54**	PHT	
NGP	92.81	19.7–132.0	23.80	53.2	31.51	9.9	43.5	13.8	0.30	−0.15	−0.30	NGP
WGH	2.40	1.7–3.0	13.18	34.8	0.81	19.6	3.2	46.2	0.43**	0.04	0.24	−0.32**
Eigenvalues	–	–	–	–		1.9	1.3	1.08	–	–	–	–
% Variation	–	–	–	–		38.5	26.5	21.6	–	–	–	–

**Significant at 5% probability level; PC, principal components; CV, coefficient of variation; GA, genetic advance; SPY, single plant yield (g); PNL, panicle length (cm); PH, plant height (cm); NGP, number of grains per panicle; HSW, weight of 100 grains (g).

Table 6. Putative association of microsatellite marker loci with quantitative traits by LD mapping.

Trait	Locus	Chromosome	Position (cM)	*P* adj.	R^2	Previous reports
Panicle length (cm)	RM302	1	147.8	0.000	18.46	Subashri *et al.* (2009)
	RM16	3	131.5	0.005	14.16	–
	RM274	5	126.6	0.003	14.07	–
	RM11	7	47.0	0.008	13.85	–
	RM311	10	25.2	0.007	14.07	Thomson *et al.* (2003)
Weight of 100 grains (g)	RM256	8	101.5	0.025	18.63	Subashri *et al.* (2009)
Grain yield per plant (g)	RM302	1	147.8	0.041	24.47	Kanagaraj *et al.* (2010)
	RM303	4	116.9	0.000	35.81	Swamy *et al.* (2011)
	RM287	11	68.6	0.010	20.41	Zhao *et al.* (2010)

RM303 and *RM287*) with grain yield per plant (table 6), located on chromosomes 1, 4, and 11, respectively. *RM303* explained maximum phenotypic variation for plant yield (35.81%) followed by *RM302* (24.5%) and *RM287* (20.4%). There were five markers associated with panicle length distributed over chromosomes 1, 3, 5, 7 and 10, of which *RM302* on chromosome 1 had described 18.5% of the phenotypic variability. *RM256* on chromosome 8 was found associated significantly with weight of 100 grains, explaining 48.6% of phenotypic variability.

Discussion

Although, subspecies level differentiation has occurred in rice during evolution, there exist residual overlaps of key genetic differences in primitive landforms. Landraces are old cultivated genotypes that have not been bred through strict breeding principles, therefore can be relatively closer to such primitive forms. Useful genetic variation in the landforms can therefore be used more judiciously in breeding programmes without narrowing down the existing genetic variation in the cultivated germplasm. In the present study, we have tested this hypothesis in a small set of un(der)exploited landraces of Tamil Nadu, India, where rice cultivation has been in practice for thousands of years. Ubiquity of the SSR polymorphism in the rice genome offer unique opportunity of studying rice genotypes for phylogenetic and evolutionary comparisons and tagging of genetic loci associated with quantitative and qualitative traits. Some of these markers are more specific to subspecific genomes than the other.

The study revealed that the proportion of biallelic markers was more when compared to triallelic and tetraallelic, and the biallelic markers showed better resolution and diversity in differentiating genotypes both between and with the subspecies, implying that the genotypes under the study are well evolved, and differentiated. To understand the discrimination power of the marker alleles, we used a new index which we called allele discrimination index (D_m) computed as the average absolute difference in allele frequencies of a marker among *indica* (*i*) and *japonica* (*j*) to provide an indication how different markers differentiated the two major subspecies. Allele discrimination was found better for biallelic markers, which got reduced when allele numbers increased. Those markers which showed very low value for the index implied that the alleles of this marker are widely and randomly distributed among *indica* and *japonica* subtypes, therefore showing less precision in subspecific differentiation. To our knowledge, this index is being used for the first time in diversity analysis. Those markers which showed high value of the D_m were appeared to be diagnostic to rice subspecies (Ni *et al.* 2002). Further, some markers (*RM170*) produced more population specific alleles enabling better differentiation of subpopulations, implying that some genomic regions were more variable between populations. Coburn *et al.* (2002) suggested that those markers that were highly variable at both the inter-subspecific and intra-subspecific levels, makes them very useful for distinguishing closely related genotypes. These alleles could be originated from the common ancestry and carried along with the partially differentiated gene pool of *indica–japonica* germplasm along the evolutionary timeline. Striking differences were observed in allele diversity between chromosomes implying that subspecies level differences do exist between chromosomes. These observations on genomewide subspecific diversity of microsatellite markers were in correspondence with earlier findings of Temnykh *et al.* (2000) and Coburn *et al.* (2002) in rice and Tenaillon *et al.* (2001) in maize. There are few other studies describing the chromosome-specific subspecies differentiation in rice (Ni *et al.* 2002; Jain *et al.* 2004; Gao *et al.* 2005; Thomson *et al.* 2007). Published analysis of rice global germplasm identified that 50% of the *indica* accessions had over 95% shared ancestry (the rest were between 65 and 95%) while 45% of the tropical *japonicas* had over 95% shared ancestry, with the remainder sharing between 62 and 95% (Garris *et al.* 2005). In this study, we have found that 17 alleles out of 237 occurred exclusively in *japonica* genotypes alone, indicating 92.8% of shared ancestry between *indica* and *japonica* genotypes.

Both the analyses of genotype clustering and population structure produced similar results, ascertaining the efficiency of the random picked panel of SSR markers in diversity assessment. Although the genotypes were absolutely differentiated at subspecies level, determination of subpopulation level within *indica* genotypes varied, between two subpopulations as revealed by analysis of population structure and

four clusters in dendrogram developed using NJ of simple matching coefficient. This is not unexpected, because the dendrogram based clustering was based on empirical groupings, either by dissimilarity values or by bootstrapping consistency. The model-based approach in population structure analysis provide better grouping (Goldstein 1991). The subpopulation containing the landraces had maximum F_{ST} value suggesting a distinct population structure (Podolsky and Holtsford 1995) for this group of landraces. This distinct group of *indica* genotypes could be ancient varieties, adapted to local niches, with conserved genetic constitution that favoured their distinct grouping from the cultivars (Ram *et al.* 2007). One of the landraces, Poongar, however was found to share more alleles with *indica* cultivars, a possible indication of its widespread use in rice breeding programmes mainly for drought tolerance. Moreover, Poongar is reported to be a good general combiner (Gomez *et al.* 2003), and is included in the lineage of three IRRI rice lines IR11817, IR11853 and IR11889 (IRRI 1985). It is well known that modern rice cultivars share a relatively narrow genetic background, when compared to the unexplored variability existing in rice germplasm worldwide. For example, the pedigree of almost all IRRI rice varieties can be traced back to few Indian landraces such as Kitchili Samba, Vellaikar, Tadukan, Thekkan and Eravaipandi (Khush and Virk 2005). Therefore, landraces of distinct genetic structure are a good promise for the future rice crop improvement.

Information on subpopulation specificity would help us in exploring the SSR markers that particularly defined a subgroup, because population specific alleles may lie in close proximity with genes that defined the characteristic phenotype of that group. Moreover, marker based differences may prove useful for the identification of introgression in progeny derived from interpopulation crosses (Coburn *et al.* 2002). We have found that the landraces remained distinct from rest of the *indica* genotypes on account of seven population specific alleles produced by markers, *RM220*, *RM279*, *RM301*, *RM305*, *RM169*, *RM170* and *RM336*. The deviation of Poongar from other landraces occurred on account of five marker alleles of *RM25*, *RM209*, *RM216*, *RM217* and *RM256*.

There are varying reports of LD patterns in rice, reported by various workers. Olsen *et al.* (2006) and Mather *et al.* (2007) reported LD decay occurring at about 1 cM distance, while Agrama *et al.* (2007), Agrama and Eizenga (2008) and Jin *et al.* (2010) reported LD decay at 20–30 cM distances using SSR markers. In the present study we have observed LD decay occurring in 10–30 cM distance with a sharp decay occurring between 10 and 20 cM (figure 3). The variation in LD pattern across chromosomal regions observed both at population and subpopulation levels suggests that the extent of LD varies among different genomic regions (Mather *et al.* 2007) and among different rice accessions (Agrama and Eizenga 2008; Jin *et al.* 2010). LD pattern of the landraces included in the present study was different, indicating that they had different types of conserved LD blocks than those of the test varieties of *indica* and *japonica* subgroups. This is evident from the considerable amount of LD decay found among the landraces (0.9%) distinctly lower than the 6% LD observed when all *indica* genotypes were put together. This little residual LD could be indicative of geographical niche limited adaptation, that enables genome conservation for generations without perceptible modifications. If true, this further indicates the need for using a dense set of markers for characterizing this subpopulation.

As expected, the phenotype data fail to capture exact genetic differences because of a high level of shared genome, manifestation of environmental influence, and relatively few number of such traits used in this study. Nevertheless, a comparison between genotype and phenotype dissimilarity matrices (Mantel *t*-test, $t = 2.58$; prob. random $Z <$ observed Z, $P = 0.995$; matrix correlation, $r = 0.26$) showed low but significant association. This may be of little significance, because of the coverage of the genome in the present study is not full, and the traits studied are not comprehensive to reveal various phenotype differences among the entries.

LD mapping (association mapping) is an approach that exploits naturally occurring haplotype blocks that are conserved in the germplasm (Malysheva-Otto *et al.* 2006; Rostocks *et al.* 2006). These haplotype blocks are in LD and their sizes vary depending on the pollination behaviour, geographical isolation, evolutionary time gaps, mutation, selection and genetic drift (Gupta *et al.* 2005). Thus, in self-pollinated crops like rice, large stretches of haplotype blocks extending over several cM are expected (Abdurakhmonov and Abdukarimov 2008). Further, the extend LD decay decides the conservation pattern of LD blocks, and larger the LD blocks, genome-wide LD mapping is possible using such set of genotypes. In the present study, although the landraces had limited LD blocks conserved among themselves, putting them together in a set of genetically wide materials have brought out LD blocks that are shared in the rice gene pool, to a sufficient level that could be utilized for genome-wide association mapping. In this approach, the subpopulation structure derived ancestry coefficients were used as covariates in the model that predicted marker–trait association to avoid spurious associations (Pritchard and Rosenberg 1999; Pritchard *et al.* 2000).

The structured association mapping revealed nine quite interesting marker–trait associations for three agronomic traits, of which six were already reported by QTL mapping approaches by various authors (table 6). This approach could identify markers with pleiotropic effects (*RM302* of chromosome 1 was found associated with both panicle length and plant yield), as well as markers that were identified to be epistatic, indicating that population wide analysis served as an effective tool in deciphering marker–trait associations.

To conclude, we could observe substantial genetic diversity and clear population structure using an un(der)exploited set of landraces, allowing realisable genome-wide association mapping in the present study, in spite of using a limited number of genotypes and markers. Although this may invite criticism of insufficiency, the results obtained here have

been nevertheless encouraging. However, a further study involving a larger group of landraces and markers is required to underpin this argument. Since the landraces included in this study are known to possess desirable attributes like resistance to abiotic factors, they qualify as suitable parental choice for varietal development in *indica* rice.

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