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Assessment of genetic diversity and relationship of coastal salt tolerant rice accessions of Kerala (South India) using microsatellite markers

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ABSTRACT: Rice (*Oryza sativa* L.*)* is one of the most significant cereal crops, about 3 billion people, nearly half the world's population; depend on rice for survival and it offers up to 80% of daily energy intake in most of the Asian countries. Knowledge of the distribution, extent and pattern of genetic variation is useful for estimation of any possible loss of genetic diversity and its role in breeding programs. This work assessed the genetic diversity among 25 coastal rice populations of five regions of Kerala (South India) using 18 microsatellite markers. A mean PIC value of 0.37 and an average of 3.5 alleles per loci were observed. Mean Heterozygosity value of 0.29 and gene diversity value of 0.41 was attained. AMOVA demonstrated that genetic differentiation was significant at $P< 0.001$ and F_{ST} index value of 0.035 was obtained. Of the total diversity, 57.76% was attributed within individuals, 38.71% was attributed among individuals within populations and 3.53% among populations. Information regarding the amount of genetic variations in these salt tolerant coastal accessions and genetic relationship between genotypes are essential for designing effective breeding programs. Especially, to meet the differentiated goals of plant breeding such as breeding for increasing yield, wider adaptation, desirable quality, pest and disease resistance.

KEYWORDS: AMOVA, Genetic Diversity, Heterozygosity, Microsatellite markers.

INTRODUCTION

Rice cultivation in the coastal regions of Kerala is the oldest cultivation practice in India, which has history of about 3000 years. In coastal areas of Kerala, farmers favor rice cultivation in the low saline phase of the production cycle, in the period of June and October and later followed by prawn farming during the high saline phase between November and April (21). In 1972, Rice research station, Vytila made a significant move to evolve Saline tolerant high yielding, breeding rice cultivars suitable for the coastal saline lands of Kerala (22). Until now, eight high yielding salinity tolerant rice varieties (VTL 1 to VTL 8) were released from this station. The landraces are genetically flexible due to their genetic variability. Since, these landraces adapt to local field conditions and they can adapt to changing environments, farming practices, and specific uses, thus it's been preferred by the farmers (25). Farmers associated with rice cultivation in Kerala cultivate both salt tolerant rice and breeding lines simultaneously.

Molecular markers have always been a dominant tool in the estimation of genetic variation and among these molecular markers, microsatellites are more chosen among the PCR based markers (9, 10). In rice, SSR markers are more popular compared to other various PCR based markers, because they are very informative, commonly mono locus, easily evaluated and cost effective (18). As SSR's are abundant and inherently potential for variation, they have been a valuable genetic marker (23, 24). In the coastal regions of the tropics, salinity is major problem for rice

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farming. Genetic relationship among 24 salt tolerant genotypes and coastal landraces of Bangladesh, were evaluated using SSR markers (19). Genetic variation of rice genotypes in Brunei Darussalam and assessment of their salinity tolerance was evaluated using SSR markers (13). Nantawan *et al.,* (11) conducted genetic diversity studies on thirty salt tolerant rice genotypes using both RAPD and SSR markers. Knowledge on salt tolerance ability delivers useful information in plant breeding programs, in order to select best parents with diverse genetic background prior to intercrossing (6).

The main objective of this study is to understand the genetic diversity of the salt tolerant rice populations of the region. This microsatellite database will be useful for rice breeders in identifying desirable traits and selection of appropriate parents in rice breeding programs. This baseline data also helps in generating a suitable practical method in salt tolerant local landrace management.

MATERIALS AND METHODS

Sample Collections

After an extensive survey of Coastal rice cultivation, five locations (Thoppumpady-Palluruthy, Kumbalanghi, Chellanam, Kadamakudy and Varapuzha) were selected as sampling sites, where rice was cultivated by farmers for about three decades. In each location 5 populations were sampled, *i.e.* five rice fields with a distance of at least one km from each other were randomly selected for sampling within each region. All individuals from the same field were regarded as members of one population (Table 1). Breeding lines grown by the farmers from the same region were also sampled (Table 2). Sampling method followed was similar to that illustrated by Qianjin *et al.* (17).

SSR Assay

DNA was extracted from rice grains by the method of Pal *et al.,* (14). 18 SSR markers covering all the twelve chromosomes were selected for the genetic diversity study based on rice genes database www.gramene.com (23, 3). Polymerase chain reaction (Eppendorf, Hamburg) amplification using SSR markers was performed in a 25 µL reaction volume which contained 25 ng of template DNA, 0.3µM of each forward and reverse primer, 250mM of each dNTPs, 1X PCR buffer (50mM KCl + 10 mM Tris HCl, pH-8.3), 2.5 mM $MgCl_2$ and 1 U of *Taq* DNA Polymerase. Amplification was performed using the following conditions denaturation at 94^0C for 5 minutes; followed by 30 cycles of one minute at 94^0C; one minute annealing at 55^0C; one minute extension at 72^0C and a final extension of 72^0C for 5 minutes. PCR amplified products were separated on 3.5% agarose gel, run at a constant voltage of 120 volts for 3h, with ethidium bromide staining (2, 4, 16). Digital images of the stained gels were captured after drying under Ultra-violet light. The band size of the amplified products was estimated using 100bp DNA ladder (Sigma).

Table 1: List of Coastal rice population's samples collected from five different locations, used in this study

Location	Population Code
Thoppumpady - Palluruthy	TP01,TP02,TP03,TP04 and TP05
Kumbalanghi	KUP01, KUP02, KUP03, KUP04 and KUP05
Chellanam	CP01,,CP02,CP03,CP04 and CP05
Kadamakudy	KAP01, KAP02, KAP03, KAP04 and KAP05
Varapuzha	VP01,VP02, VP03, VP04 and VP05

Data analysis

The amplified SSR DNA bands indicating different alleles were scored as homozygous genotypes (AA, BB, CC) or heterozygous genotypes (AB, AC, BC). Heterozygosity or which is often termed as observed heterozygosity (*Ho*), expected heterozygosity (*He*)/ gene diversity and allele number for each microsatellite marker was calculated using POWERMARKER V3.0. Genetic relationships among various Coastal rice populations and elite cultivars were plotted on a dendrogram based on *Nei's* genetic distance matrix (12), by means of unweighted pair group method with arithmetic mean (UPGMA). Analysis of Molecular Variance (AMOVA) was used to partition SSR variation among individuals, among individuals within populations and among populations. AMOVA and F_{ST} indices were calculated using ARLEQUIN v3.5.

RESULTS

In this study, a total of 59 alleles were detected by the 18 microsatellite loci with an average of 3.2 alleles per locus.A minimum of 2 alleles were observed in RM 184, RM 220,

RM 226, RM 240, RM 421 and maximum of 7 alleles were observed in RM 231. Highest gene diversity value was spotted at 0.7336 at RM 231 locus and lowest at locus RM 220 (0.1609) (Table 3). Genetic diversity was higher at *thoppumpady-palluruthy* region (*He*=0.4592) and lowest at the Kadamakudi region (*He*=0.3370). The observed heterozygosity (*Ho*) was higher in Varapuzha populations (0.32500). The UPGMA dendrogram based on *Nei's* genetic distance matrix displayed that all the coastal rice populations and breeding lines were clustered closely into a large group. Within this large group two sub-groups were clearly plotted. In the large group breeding varieties such as VTL-3, VTL-7, VTL-8 and VTL-5 were seen relatively distant from the two sub-groups (Figure 1). The elite varieties such as VTL-4 and VTL-6 exhibited close relationship to coastal rice populations of the region kumbalanghi (KUP05) and varapuzha (VP05, VP04 and VP01). The rice populations of Chellanam (CP03 and CP04) and Kadamakudi (KAP04) regions showed close genetic relationship with breeding lines VTL-1 and VTL-2. For further knowledge of genetic relationship, Principal Component Analysis (PCA) was conducted (Figure 2). PCA further confirmed the results exhibited by UPGMA dendrogram. In PCA analysis also, elite varieties (VTL-3, VTL-5, VTL-7 and VTL-8) were seen scattered and separated from the two sub groups.

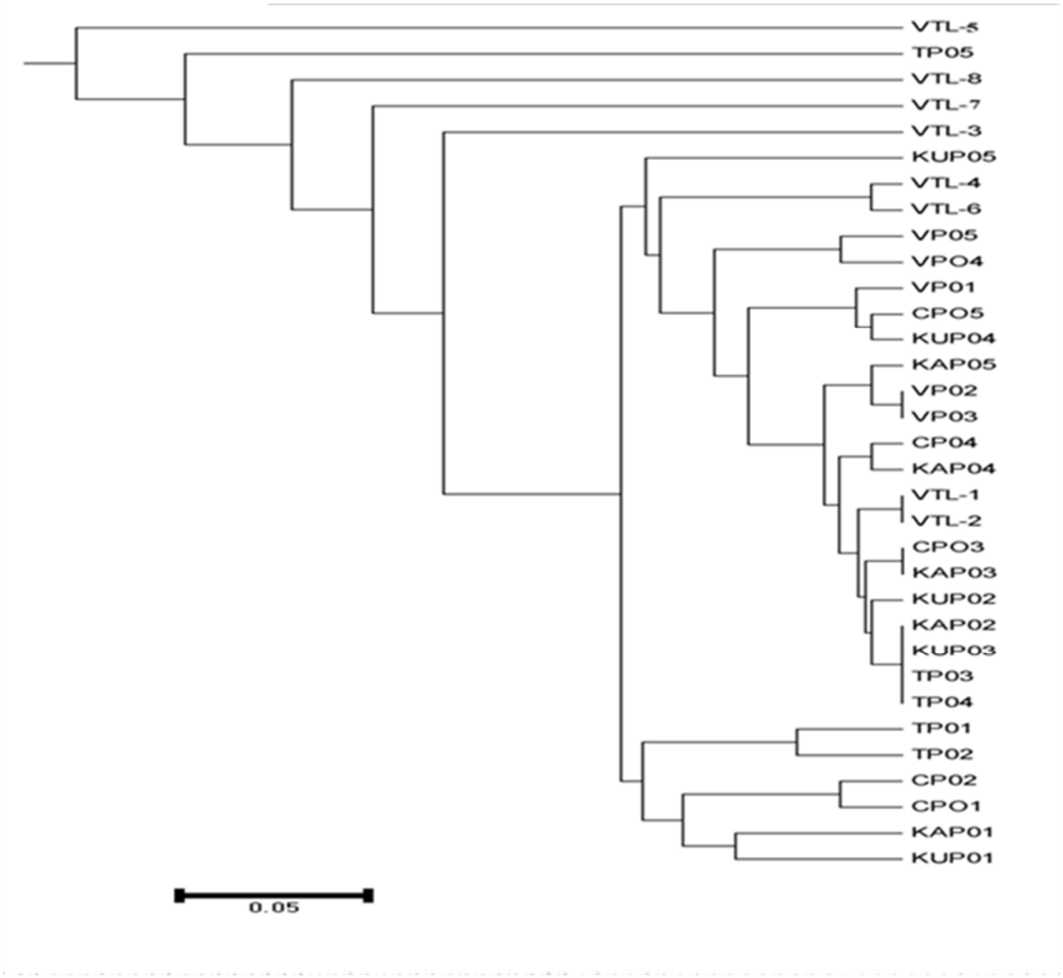

Figure 1: Dendrogram constructed on the basis of 18 SSR loci in 25 *pokkali* rice populations and breeding (VTL) varieties using *Nei's* genetic distance coefficients. Codes for the coastal rice populations and breeding (VTL) varieties are given in Table-1 and 2.

Table 2: List of elite rice varieties used in this study, along with their parental relation

Name of the elite rice varieties	Parental lines / Parental relations
VTL-1	*Chootu Pokkali*
VTL-2	*Cheruvirippu*
VTL-3	VTL-1 / TN-1(Taichung Native 1)
VTL-4	*Chettivirippu* / IR4630-22-2-17
VTL-5	*Mahsuri* Mutant
VTL-6	*Cheruvirippu* / IR 5
VTL-7	IR8 / Patnai 23
VTL-8	IR47310 / CSR-10 9441

Two coastal rice populations of *thoppumpady-palluruthy* and chellanam regions were also clustered together. The PCA analysis clearly confirmed that rice populations exhibited close relationship to certain breeding lines (VTL-4, VTL-6, VTL-1 and VTL-2) cultivated by farmers. Analysis of molecular variance (AMOVA) calculated using Arlequin v3.5.1.3 produced a high genetic diversity among individual accessions. Total diversity was highest within individuals, providing 57.76% variation and 38.71% variation among individuals within populations.

DISCUSSION

Rice germplasm richness is very high in the Indian subcontinent region that includes, landraces, wild *Oryza* species, hybrids which are naturally generated between the cultivars, wild relatives and the germplasm resources created in the breeding programs (20). In this study a total of 59 alleles were detected. Also, an average of 3.2 alleles per locus was detected. A maximum of 7 alleles were observed in RM 231 and highest gene diversity value was observed at thoppumpady-palluruthy region (*He*=0.4592). Similar results were produced in genetic population study on weedy rice found in north-eastern China (17). Distribution of genetic variation within landrace population is influenced by natural processes like drift, selection, gene flow and hybridization. But, in these coastal rice populations we expect major influence of factors such as seed exchange, selection by farmers and cultivation practices because rice populations in these part of India is a domesticated species. Thus, the reason for the above results might be the application of different farming practices by

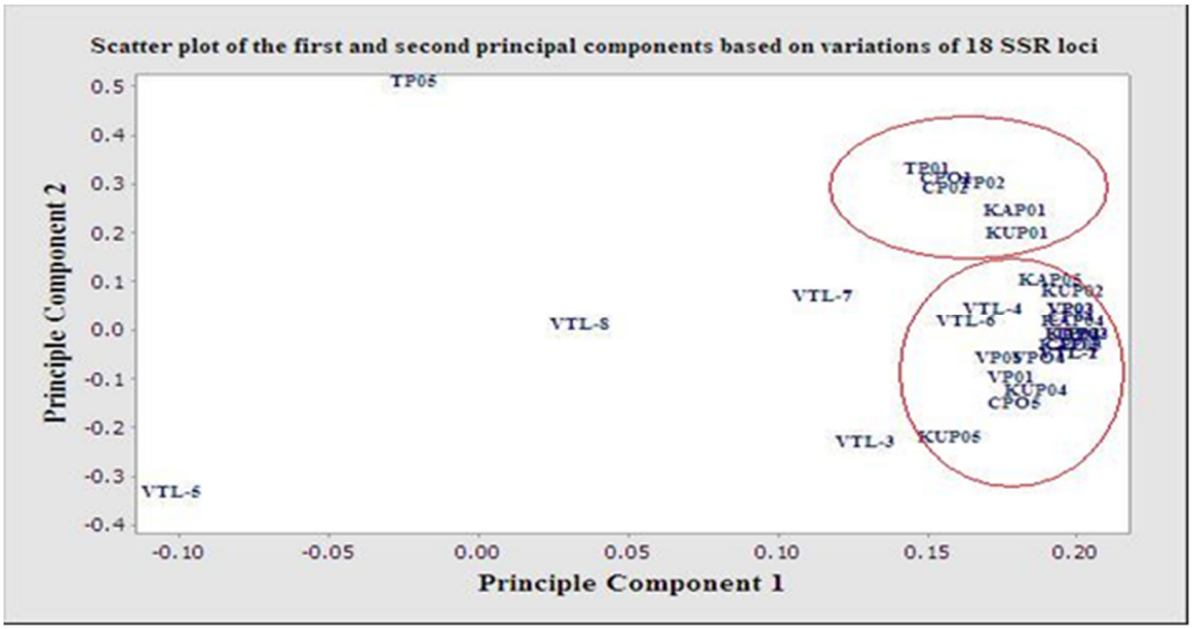

Figure 2. Principal Component Analysis (PCA) showing variation of 18 SSR loci of Coastal rice populations and breeding lines. Two sub-groups have been circled.

Table 3. List of 18 SSR markers used in this study, indicating repeat motifs, chromosome position, primer sequences, expected heterozygosity (*He*) and Allele number (*Na*)

Primer Code	Chromosome Location	Repeat Motifs	Primer sequence (Forward)	Primer sequence (Reverse)	Gene Diversity / Exp. Heterozygosity(*He*)	Allele No.(N_a)
RM 6	2	(AG)16	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCA	0.5415	3.0
RM 19	12	(ATC)10	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	0.4516	3.0
RM 25	8	(GA)18	GGAAAGAATGATCTTTTCA	CTACCATCAAAACCAATGTT	0.2578	3.0
RM 44	8	(GA)16	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC	0.6021	3.0
RM 164	5	(GT)16TT(GT)4	TCTTGCCCGTCACT	GCAGCCCTAATGCT	0.5484	5.0
RM 170	6	(CCT)7	TCGCGCTTCTTCCT	CCCGCTTGCAGAGG	0.3875	3.0
RM 184	10	(CA)7	ATCCCATTCGCCAA	TGACACTTGGAGAG	0.2509	2.0
RM 189	9	(AG)11	GGGAGTTGAAGTGG	CACGCGACTTCAGT	0.2128	3.0
RM 206	11	(CT)21	CCCATGCGTTTAAC	CGTTCCATCGATCC	0.5035	3.0
RM 220	1	(CT)17	GGAAGGTAACTGTT	GAAATGCTTCCCAC	0.1609	2.0
RM 224	11	(AAG)8(AG)13	ATCGATCGATCTTCACGA	TGCTATAAAAGGCATTCG	0.4585	3.0
RM 226	1	(AT)38	AGCTAAGGTCTGGG	AAGTAGGATGGGGC	0.2076	2.0
RM 231	3	(CT)16	CCAGATTATTTCCTGAGG	CACTTGCATAGTTCTGCA	0.7336	7.0
RM 240	2	(CT)21	CCTTAATGGGTAGT	TGTAACCATTCCTT	0.2076	2.0
RM 248	7	(CT)25	TCCTTGTGAAATCT	GTAGCCTAGCATGG	0.6228	5.0
RM 261	4	C9(CT)8	CTACTTCTCCCCTTGTGT	TGTACCATCGCCAAATCT	0.5121	5.0
RM 337	8	(CTT)4-19-(CTT)8	GTAGGAAAGGAAGGGCAG	CGATAGATAGCTAGATGT	0.2993	3.0
RM 421	5	(AGAT)6	AGCTCAGGTGAAAC	ATCCAGAATCCATT	0.4567	2.0

farmers. Application of different farming practices by rice farmers from their predecessors was stated by a senior rice farmer (15). In order to understand the genetic relationship of rice populations to the other breeding lines cultivated by farmers, (12) *Nei's* genetic distance matrix was calculated. To illustrate genetic relationship among various groups, *Nei's* UPGMA dendrogram based on genetic distance was constructed. Dendrogram showed considerable genetic differentiation among Coastal rice populations among regions. Two *thoppumpady-palluruthy* (TP01 and TP02) and two *chellanam* (CP01 and CP02) populations were clustered in a sub-group, separated from other populations of that region. The population TP05 was also seen distantly located along with elite varieties. PCA also confirms the results, displayed by the *Nei's* UPGMA dendrogram.

AMOVA indicated that total diversity was highest within individuals, providing 57.76% variation and lowest among populations, giving about 3.53% variation. The F_{ST} index was 0.035, which according to Kiambi *et al.,* (7) and Kitavi *et al.,* (8) suggest little differentiation at population level. Similar results were obtained in other landrace studies that differentiated red rice land race *Chuhatu* and *Katheri* from Himachal Pradesh (5) and another study on Thai rice (25).

Variations at the individual level indicate the presence of certain new alleles or combination. These new alleles or combinations are expected to arise and their frequency increases under farmer's management. These alleles which arise at the expense of other alleles will gradually replace them. As fit alleles or combinations enjoy selective advantage, other alleles will be less fit and decline, thus results in evolutionary substitution (1). In traditional agricultural systems, farmers and local farming communities have been major contributors in conservation of crop genetic diversity. They uphold the process of crop evolution and adaptation. But, diversity in farmer varieties is dependent to an extent on population size and the environmental conditions; also, the seed sources (17).

In conclusion, microsatellite marker based characterization of coastal rice collections revealed that variations exist among individual accessions and the pattern of genetic diversity varied among regions. A mean PIC value of 0.37 and an average of 3.5 alleles per loci were observed. Mean Heterozygosity value of 0.29 and gene diversity value of 0.41 was attained. The F_{ST} index was 0.035 and AMOVA indicated 57.76% diversity within individuals, 38.71% was attributed among individuals within populations and 3.53% among populations. These results confirm the influence of factors such as seed exchange, selection by farmers and cultivation practices in coastal rice population of South India. This study of coastal rice population, functions as a baseline data that can support, in checking the changes in population structure over the period of time and molecular level data in understanding detrimental evolutionary patterns. The data obtained can be potentially exploited by the rice breeders in conventional breeding programs or by molecular breeders/researchers in identifying various desirable traits and selection of suitable parents for their use in rice improvement.

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ارزیابی تنوع ژنتیکی و روابط خویشاوندی ارقام برنج متحمل به شوری در جنوب هند (منطقه کرالا) با استفاده از مارکرهای ریز ماهواره

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چکیده

برنج (*Oryza sativa* L.) یکی از مهمترین گیاهان زراعی است که حدود سه میلیارد نفر از جمعیت دنیا برای زنده ماندن به ان وابسته بوده و۸۰ درصد انرژی مورد نیاز روزانه در اکثر کشور های آسیایی از آن تامین می شود. اگاهی از نحوه توزیع گسترده و الگوی تغییرات ژنتیکی برای تخمین هر نقصان احتمالی تنوع ژنتیکی و نقش ان در برنامه های اصلاحی سودمند می باشد. در تحقیق حاضر تنوع ژنتیکی میان ۲۵ جمعیت برنج ساحلی پنج منطقه از کرالا (در جنوب هند) با استفاده از ۱۸ مارکر ریزماهواره مورد ارزیابی قرار گرفته است. میانگین مقدار PIC برابر با ۰/۳۷ و میانگین ۳/۵ آلل برای هر جایگاه مشاهده شد. میانگین مقدار هتروزیگوسیتی برابر با ۰/۲۹ و ارزش تنوع ژنی ۰/۴۱ بدست آمد. تجزیه تحلیل اختلاف مولکولی (AMOVA) نشان داد که تفاوت ژنتیکی در سطح $p<0.001$ معنی دار بوده و مقدار شاخص F_{ST} برابر با ۰/۰۳۵ می باشد. از مجموع تنوع، ۵۷/۷۶ درصد در میان افراد و ۳۸/۷۱ درصد بین جمعیت ها مشاهده گردید. اطلاعات بدست آمده نشان دهنده میزان تنوع ژنتیکی در این ارقام متحمل به نمک و روابط خویشاوندی ژنتیکی میان ژنوتیپ‌ها می‌باشد. برای طراحی برنامه های اصلاحی موثر ضروری بوده و برای دستیابی به اهدافی مانند اصلاح برای افزایش عملکرد، تطبیق‌پذیری گسترده تر، کیفیت مطلوب‌تر و مقاومت به آفات و بیماری ها می‌توان از آنها استفاده کرد.

کلمات کلیدی: AMOVA، تنوع ژنتیکی، هتروزیگوسیتی، مارکرهای ریز ماهواره