MOLECULAR DIVERSITY IN RICE GENOTYPES DIFFERING IN PHYSIOLOGICAL MECHANISMS OF SALT TOLERANCE THROUGH SSR AND ISSR MARKERS

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ABSTRACT : Two different DNA-based techniques *viz,* simple sequence repeat (SSR) and inter simple sequence repeat (ISSR) markers were used to estimate genetic diversity among 20 rice genotypes possessing different physiological mechanisms contributing to salt tolerance. A total of 11 clear and repeatable bands were amplified from ten selected SSR primers pairs and 43 fragments were detected from nine ISSR primers. The level of polymorphism was 1.1% with SSR compared to 90.7% with ISSRs. Mean genetic similarity of 0.88 based on SSRs and 0.85 using ISSRs was observed. A total of 43 (39 polymorphic) and 11 bands were detected using 9 ISSR primers and 10 well distributed mapped SSR markers, respectively. Estimates of genetic similarity of ISSRs based on the 39 polymorphic markers between 20 rice cultivars ranged from 0.55 for PR108/CSR19 to 0.94 for Pokkali/CSR20 with an average of 0.81. The estimates revealed by the 11 polymorphic SSR bands showed the average value (0.94) and also the range of genetic similarity (from 0.86 to 1.00 for CSR22/CSR18 and CSR24/CSR20, respectively) reflecting their hyper variability and their high resolution power. The findings are likely to expedite breeding new salt tolerant cultivars by involving parents from diverse molecular clusters.

Keywords: Salt tolerance, Dendrogram, ISSR and SSR markers, Restorers, Maintainers, Molecular diversity, Physiological mechanism.

INTRODUCTION

Rice is one of the economically and nutritionally important cereals in the world and is the principal staple food in Asian countries. Numerous studies are available and documented on genetic diversity and thus amply utilized for genetic improvement of this cereal crop. The complete deciphering of rice genome sequence, the first in any cereal crop has opened up new vistas in its genetic improvement through molecular markers approach. Soil salinity affects about 1000 million hectares land globally and therefore poses a formidable task of taking up agriculture and enhancing productivity in these areas. About 100 million ha in South and South-east Asia are covered by problem soils where rice is the staple crop. Salt tolerance is a polygenic trait being controlled by different physiological mechanisms and amenable to genetic improvement. In the Indian context, Singh et al (2004 a) have thoroughly reviewed the progress and future thrust areas of harnessing biological phenomenon of salt tolerance in enhancing agricultural productivity. Salt tolerance is controlled by different mechanisms like Na exclusion, K mining ability, lower Na/K ratio, low Cl uptake, tissue tolerance and higher growth vigour, donors for these traits have been identified (Singh et al, 2004). It is imperative to pyramid all these traits together in a single genotype to achieve a higher level of tolerance either through recombination breeding or heterosis breeding (Gautam and Singh, 2004). Micro-satellites or simple sequence repeats (SSRs) are the genetic markers that identify alleles with high reliability and reproducibility and are widely used in taxonomy and diversity studies . SSRs appear to be ubiquitous in higher organisms, although their frequency varies among species. They are generally abundant, dispersed throughout the genome, and also show higher levels of polymorphisms than other genetic markers. These are also considered as second generation markers and are subset of the tandomly repeated DNA family represented by extremely short nucleotide sequence repeats from 1-5 base pairs (bp) that are abundantly present and interspersed in eukaryotic genomes . SSRs are reported to be highly informative in plants providing many different alleles for each marker, even among closely related individuals. ISSR markers (Zietkiewicz et al., 1994) RAPD markers (Williams et al., 1990) are two molecular typing approaches that have been used to detect genetic diversity in plants. ISSR analysis has been used for cultivar identification in numerous plant species including rice (Joshi et al., 2000), apple and strawberry . ISSR markers involve the PCR amplification of DNA using single primers based on microsatellite sequences. These primers target microsatellites that are abundant throughout the eukaryotic genome.

MATERIALS AND METHODS

Plant material and DNA isolation:

A total of 20 rice cultivars possessing different mechanisms of salt tolerance which are being maintained and used at CSSRI, Karnal were selected for the present study (Table 1). The genotypes were grown in the glass house. DNA was isolated from the tissues of juvenile leaves using modified CTAB method (Saghai and Maroof et al, 1984). The resultant DNA was stored in TE buffer and concentrations were estimated by agarose gel (0.8%) electrophoresis using DNA ladder (Fermentas) as standard.

Table 1: List of salt tolerant rice genotypes and their characteristics

Genotype	Parentage	Stress adaptation limit		Physiological mechanism of tolerance	M or R For 'WA' CMS system *
		pH_2	ECe(dS/m)		
CSR8	CSR 1 Mutant	< 9.6	< 7.0	Na^+ excluder	?
CSR10	M40-431-24-114/Jaya	< 10.2	<11.0	Na^+ excluder, K^+ miner, Low Cl^- uptake, Low Na^+/ K^+ ratio	M
CSR11	M40-431-24-114/Bas.370	< 10.2	<11.0	Early vigour, K^+ accumulator	M
CSR13	CSR1/Bas.370//CSR 5	< 9.9	<9.0	Na^+ excluder	R
CSR18	RPA 5829/CSR 5	< 9.8	<8.0	Low Na^+/ K^+ ratio	P
CSR19	CSR 1/Bas.370	< 9.8	<8.0	Low Na^+/ K^+ ratio, Low Cl^- uptake, Na^+ excluder	R
CSR20	CSR 5/Palaman 579	< 9.8	<8.0	Na^+ excluder, K^+ accumulator	
CSR21	IR5657-33-2/IR4630-22-2-5-1-3	< 10.0	<9.0	Tissue tolerance to Na^+, K^+ accumulator	I
CSR22	IR64//IR4630-22-2-5-1-3/IR9764-45-2-2	< 9.9	<10.0	Na^+ excluder	R
CSR23	IR64//IR4630-22-2-5-1-3/IR9764-45-2-2	< 10.0	<10.0	Na^+ excluder, K^+ accumulator	R
CSR24	IR58/Chettiviruppu	< 9.8	<10.0	Na^+ excluder	R
CSR27	Nona Bokra/IR5657-33-2	< 9.9	<10.0	Early vigour, Tissue tolerance to Na^+	I
IR42	?			Low Na^+/ K^+ ratio	?
Swarnadhan	?			K^+ accumulators	?
Pokkali	?			Tissue tolerance to Na^+	?
Jaya	TN 1 / T141			Low Cl^- uptake	?
Panvel 123	?			K^+ accumulator	?

*M- Maintainer, *R- Restorer, *P- Partial, *I- Intra-varietal variants

SSR amplification:

The polymerase chain reaction (PCR) was carried out in a Biometra (T gradient 5.02 version) thermal cycler using 10 SSR primers (Table 2). The PCR reaction mixture (10 µl) contained 1.0 µl 10x PCR buffer, 2.5 mM $MgCl_2$, 40 µM dNTP mix, and 0.3 U Taq DNA polymerase (Fermentas). SSR primers and ISSR were obtained from (Life Science Imperial, USA.). All the reactions were carried out with different rice genomic DNA templates at the final concentration of 60 nano gram per reaction. PCR thermocycling was carried out on a single SSR primer (both reverse and forward) in each PCR reaction, which was carried out in 20 µl reaction mixture containing 30- 90 nano gram of template DNA, 100 µM of each of the four dNTPs (Fermentas), 2.0 µl 10x buffer (Fermentas), 1 unit Taq DNA polymerase, 2.5 mM $MgCl_2$ and 0.5 µM of each primer (reverse and forward). The PCR profile started with a hot start at 94^0C for 4 minute, followed by 35 amplification cycles of denaturing at 92^0C for 1 minute, annealing at 55^0C for 1 minute, extension at 72^0C for 2 minute and final extension at 72^0C for 7 minute. The PCR product were resolved by electrophoresis on 2.8 % agarose (Life Science Imperial USA).The gels were stained with ethidium bromide and then photographed under UV light inside gel documentation system (Alpha Innotech, USA). A total of ten SSR primers were utilized to know genetic diversity among twenty rice cultivars belonging to *indica* types (Table 1).

Table 2. SSR Primers, data on DNA profile and polymorphism generated using 10 SSR markers.

MARKER ID	RESOLUTION POWER	POLYMORPHIC RESOLUTION CONTENT	MARKER INDEX
RM6302	0	0	0
RM7551	0	0	0
RM7309	0	0	0
RM6133	0	0	0
RM3859	0	0	0
RM6011	0.4	0.32	0
RM1209	0.85	0.34	0.43
RM3262	1.1	0.18	0.18
RM5637	0.5	0.255	0.255
RM3859	0.4	0.095	0.095
Total	0.325	0.119	0.096

ISSR amplification:

A total of nine ISSR primers from Life Science Imperial (USA) were used for genetic diversity (Table 3). Optimal conditions for DNA amplification were empirically determined by testing different concentrations of genomic DNA of different rice genotypes on a single ISSR primer (both reverse and forward) was used in each PCR reaction, which was carried out in 20 µl reaction mixture containing 30-90 nanogram of template DNA, 100 µM of each of the four dNTPs (Fermentas), 2.0 µl 10x buffer (Fermentas), 1 unit Taq DNA polymerase, 2.5 mM $MgCl_2$ and 0.5 µM of each primer (reverse and forward). PCR amplification was performed in 96 well plates on T gradient thermal cycler (5.02 version Biometra) under a hot start at 94^0C for 4 minute, followed by 35 amplification cycles of denaturing at 94^0C for 30 seconds, annealing at 45^0C for 45 second, extension at 72^0C for 1 minute and final extension at 72^0C for 7 minutes. The PCR product was resolved by electrophoresis on 1.6 % Agarose (Life Science Imperial USA.) gels stained with ethidium bromide, photographed under UV light inside gel documentation system.

Data Analysis:

DNA profile data for each of marker systems have been presented by using the term assay unit . Assay unit is defined as one reaction involving one primer for ISSR or one set of primers for SSR. The PCR products from ISSR and SSR analyses were scored quantitatively for the presence (1) or absence (0) of bands. Only clear and unambiguous bands were scored for ISSR and SSR markers. The resultant genetic similarities between the cultivars were measured by the Jaccards's similarity coefficient based on the proportion of shared alleles using simqual sub programme of software NTSYS-pc version 2.02h. The resultant distance matrix data were used to construct dendrograms by using the un-weighted pair group method with an arithmetic average (UPGMA) sub programme of NTSYS-pc.

RESULT AND DISCUSSION

The details of polymorphism of rice genotypes are given in tables 2 and 3. In the ISSR analysis out of 11 primers screened for amplification of all the genotypes, 9 primers gave reproducible and scorable amplification products. Hence they were used for the further analysis. A total of 43 bands were obtained (average of 4.8 bands per primer) among which 39 were polymorphic (90.7%) across the 20 rice genotypes. The highest and the lowest number of polymorphic bands per assay unit was 9 and 2, respectively. In the SSR assay, a total of 11 fragments were obtained from the 10 SSR assay units. The number of polymorphic bands per assay unit ranged from 1 to 2 with an average of 1.1. An example of ISSRs and SSRs banding pattern has been shown in figure 1&3. A summary of the genetic similarity estimates between pairs of genotypes, calculated for each marker system, is shown in Table 3.

Table- 3. ISSR primers, data on DNA profile and polymorphism generated using 9 ISSR markers.

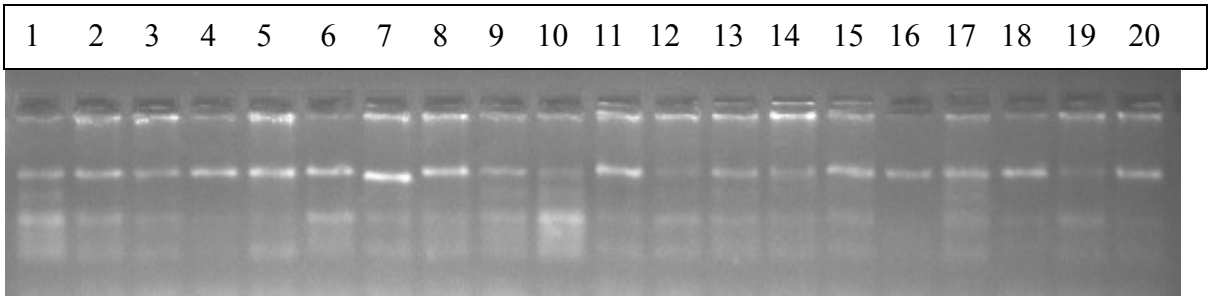
Marker ID	Total number of bands	Polymorphic band	Percent polymorphism	Resolution power (RP)	Polymorphic information content (PIC)	Marker index (MI)
844B	2	2	100.0	0.40	0.18	0.36
HB9	3	2	66.7	0.80	0.18	0.55
HB10	5	5	100.0	1.50	0.25	1.25
17899B	8	8	100.0	1.70	0.18	1.46
HB11	2	0	0.0	0.00	0.00	0.00
844A	6	6	100.0	2.60	0.29	1.75
17898B	9	8	88.9	4.10	0.31	2.81
HB12	3	3	100.0	0.80	0.20	0.61
HB15	5	5	100.0	2.00	0.29	1.47
Total	43	39	90.7	1.54	0.21	1.14

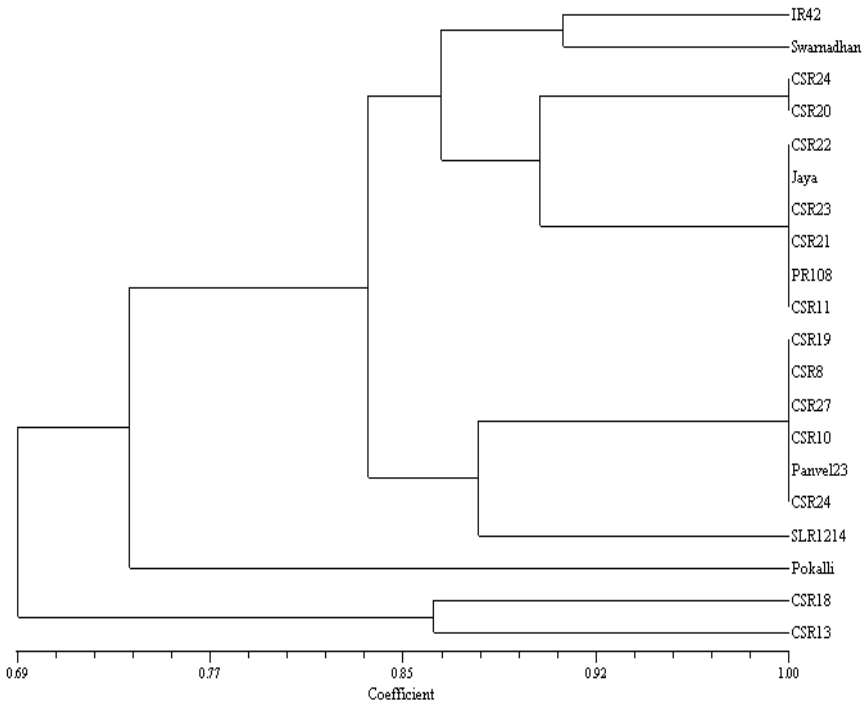
Micro satellite data gave lower average similarity than ISSRs. Estimates of genetic similarity of ISSRs based on the 39 polymorphic markers between 20 rice cultivars ranged from 0.55 for PR108/CSR19 to 0.94 for Pokkali/CSR20 with an average of 0.81. The estimates revealed by the 11 polymorphic SSR bands showed the average value (0.94) and also the range of genetic similarity (from 0.86 to 1.00 for CSR22/CSR18 and CSR24/CSR20 respectively) reflecting their hyper variability and their high resolution power. Three dendrograms were constructed to express the results of cluster analyses based on data obtained by ISSR, SSR, and ISSR+SSR amplification products(Fig.5). The dendrograms obtained with ISSR and ISSR+SSR markers were more similar to each other than to the dendrogram based on SSR. The discriminatory power of ISSR+SSR markers was evaluated by three parameters. The polymorphic information content (PIC) for each marker was calculated as proposed by Andersons et al. (1993) , as $PIC_i = 2f_i (1- f_i)$, where PIC_i is the polymorphic information content of marker i, f_i is the frequency of the marker bands present, and $(1- f_i)$, is the frequency of the marker bands absent. Each marker has aximum of 0.5 when half of the accessions have the bands and the other half does not have the band (De Rick et al.,2001). PIC was averaged over the bands for each primer. The resolving power (Rp) of the primers was calculated according to Provost and Wilkinson (1999) as $Rp=\Sigma Ib$ where Ib (band in formativeness) takes the value of 1-[(2x0.5-p), p being the proportion of the genotypes containing the band. The third parameter used was the marker index (MI) as proposed by Powell et al.(1996) and used by Milbourne et al.(1997).

MI is the product between diversity indexes (equivalent to PIC). Regarding the polymorphism, the salt tolerant rice material tested revealed different levels of genetic diversity (Table 2& 3) employing both markers ISSR (PIC-0.21) and SSR marker (PIC-0.12). Among ISSR primers 17898B was having the highest PIC value (0.31) followed by 844A (0.29) whereas amongst the SSR markers, RM1209 revealed highest genetic difference (0.34) among the accessions followed by RM6011 (0.32) . Considering the higher PIC value and marker index (MI) value it is concluded that two ISSR primers 17898B and 844A , and two SSR primers RM1209 & RM6011 can be used to estimate the genetic diversity of such rice germplasm material. Nevertheless, this indication can be confirmed with more number of genotypes and markers. At 85% similarity index for SSR markers, 20 genotypes were grouped in four major clusters. Cluster 1 consists of ten varieties while cluster 2 has seven. The cluster 3 has only one and cluster 4 consists of 2 varieties. At 70% similarity index for ISSR markers, these 20 varieties grouped in seven major clusters. Only cluster 3 contained fourteen accessions while the remaining six clusters contained one variety each. Out of fourteen varieties in cluster 3, six varieties (43%) were Na^+ excluder namely CSR8, CSR13, CSR20, CSR22, CSR23, and CSR24, while four varieties (28%) were K^+ accumulators namely CSR11, CSR21, Panvel 123 and Swarnadhan and other remaining (29%) were possessing different mechanisms imparting salt tolerance. Combined over both types of markers (SSR+ISSR) at 73% similarity index, 20 lines could be grouped in seven major clusters. In the combined case, only cluster 2 contained fourteen varieties while other six clusters contained one variety each. Almost in conformity with ISSR analysis, in cluster 2, out of fourteen varieties, six varieties (43%) were Na^+ excluder
(CSR8, CSR13, CSR20, CSR22, CSR23, and CSR24), while four varieties (28%) were K^+ accumulators (CSR11, CSR21, Panvel 123, and Swarnadhan) and other remaining (29%) possesss different mechanistic traits. It is concluded that two SSR markers RM1209 and RM6011 and two ISSR primers 17898B & 844A can be used to estimate the genetic diversity between genotypes among the salt tolerant rice germplasm tested. Efforts are underway to convert maintainers CSR10, CSR11 and PR108 into Wild Abortive CMS lines through repeated back-crossing which could be used as female parents for making potential rice hybrids by involving salt tolerance donor restorers like CSR13, CSR19,CSR22, CSR23 and CSR24. Based on the revelation of combined SSR and ISSR molecular diversity in the present study, it is suggested that the CMS lines of CSR10 should be pollinated by restorers CSR13, CSR22,CSR23 and CSR24 for getting heterotic salt tolerant hybrids. Similarly CSR11 could be used as female parent for restorers CSR13, CSR19, CSR22 and CSR23. PR108 (whose CMS line is already available as PMS11A) can be crossed with CSR19 and CSR24. As regards conventional hybridization, present analysis indicates that crossing CSR10, CSR19, CSR36 and SLR1214 with genotypes from other diverse clusters IR42, CSR24, CSR11, CSR18 and CSR20 is expected to form transgressive recombinants.

These results will be of great utility while breeding suitable salt tolerant rice cultivars by crossing parents from different molecular clusters. This will also be a guiding information for developing suitable CMS lines from these known maintainers through repeated back-crossing. Harnessing the molecular diversity in combination with combining diverse mechanisms from CMS and restorer parents will lead towards the development of salt tolerant heterotic hybrids in rice.

Figure -1. SSR profile of rice genotypes generated by primer RM6440





Lane 1: IR42, Lane 2: CSR24, Lane 3: Swarnadhan, Lane 4: CSR18, Lane 5: CSR19, Lane 6: CSR22, Lane 7: Pokkali, Lane 8: CSR20, Lane9: CSR8, Lane 10: Jaya, Lane 11: CSR27, Lane 12: CSR11, Lane 13: CSR10, Lane 14: Panvel 123, Lane 15: CSR36, Lane 16: PR108, Lane 17: CSR13, Lane 18: CSR21, Lane 19: SLR1214, Lane 20: CSR23

Figure 2: SSR dendrogram:

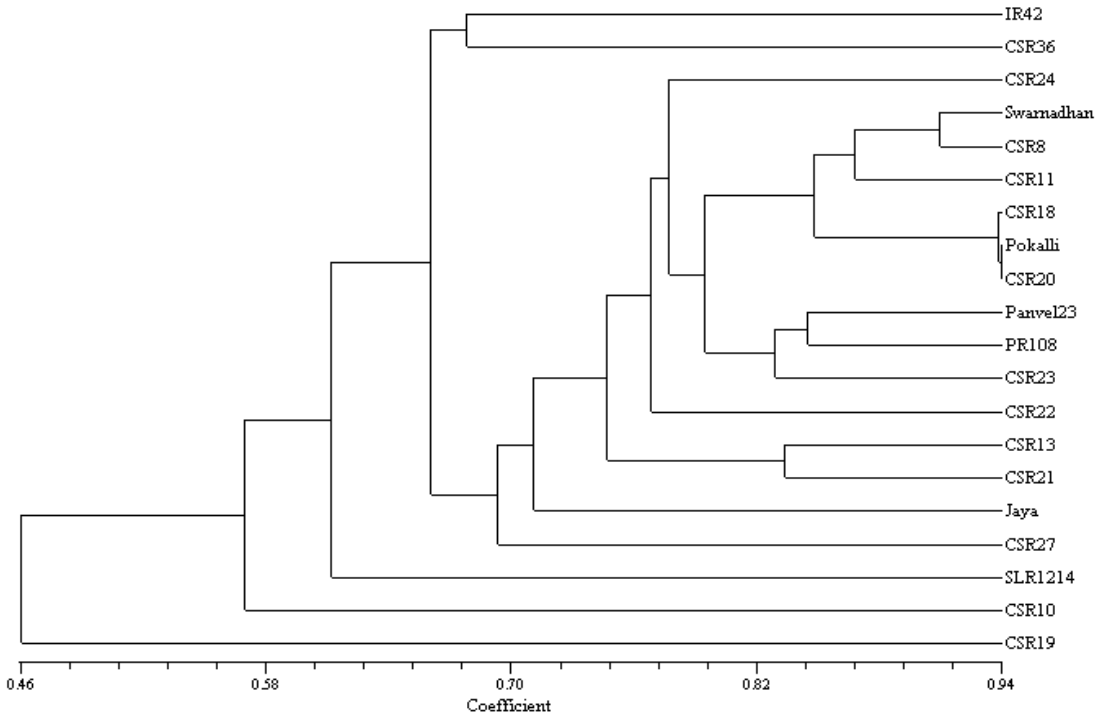


Figure-4: ISSR dendrogram

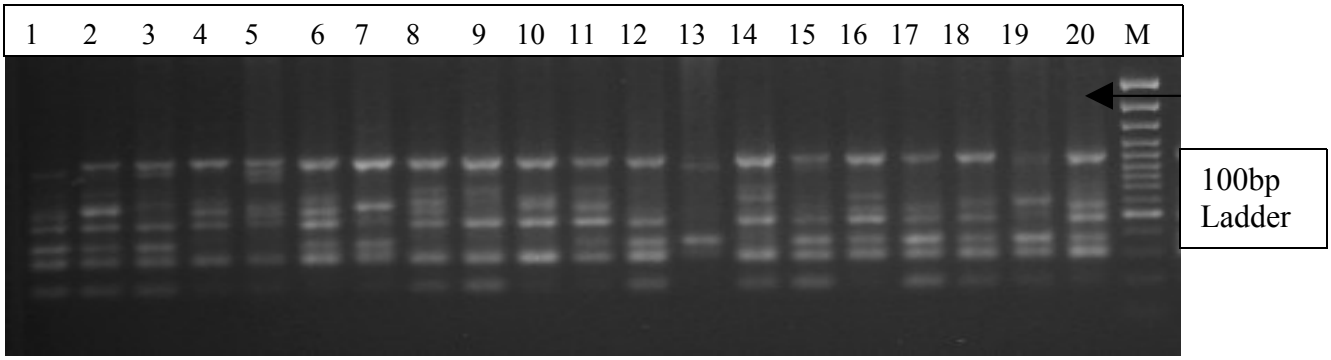


Figure-3. ISSR profile for rice genotypes generated by primer 844A

Lane 1: IR42, Lane 2: CSR24, Lane 3: Swarnadhan, Lane 4: CSR18, Lane 5: CSR19, Lane 6: CSR22, Lane 7: Pokkali, Lane 8: CSR20, Lane9: CSR8, Lane 10: Jaya, Lane 11: CSR27, Lane 12: CSR11, Lane 13: CSR10, Lane 14: Panvel123, Lane 15: CSR36, Lane 16: PR108, Lane 17: CSR13, Lane 18: CSR21, Lane 19: SLR1214, Lane 20: CSR23

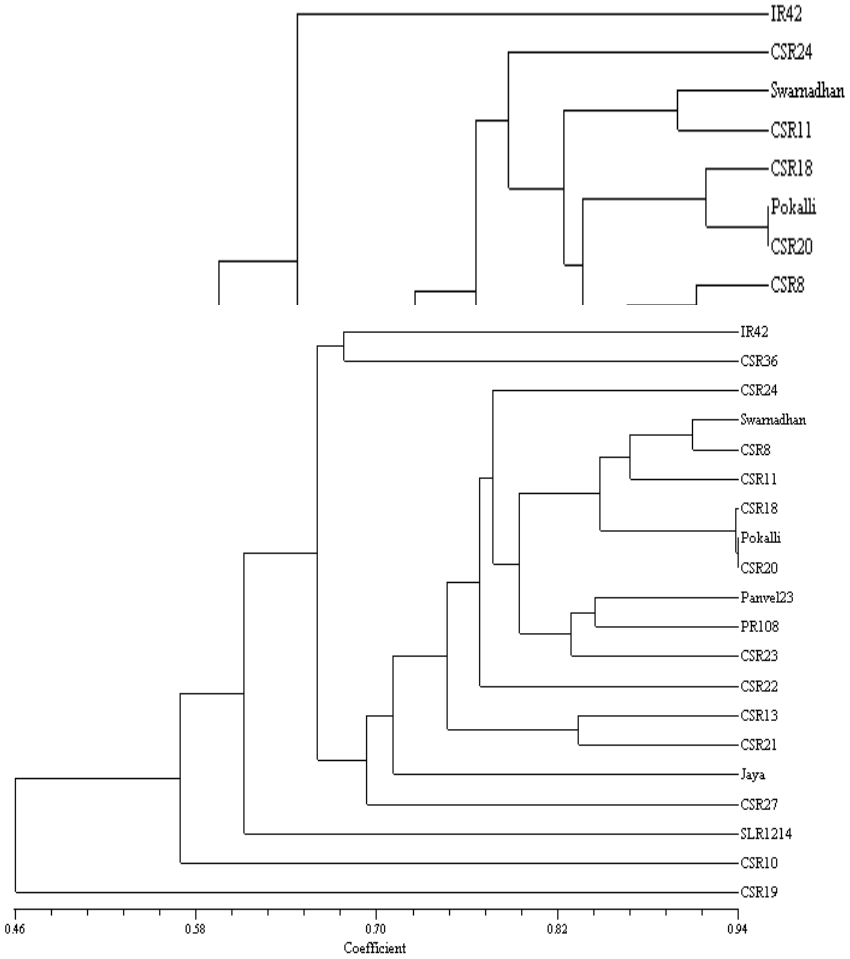


Figure 5. Pooled ISSR-SSR dendrogram

Dendrograms showing genetic relationships between rice genotypes based on genetic matrix data obtained using (figure 2) 10 SSR primers, (figure 4) 9 ISSR primers and (figure 5) pooled ISSR-SSR allelic profiles

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