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Assessment of genetic diversity of some commonly grown rice genotypes of South Bengal using microsatellite markers associated with the *saltol* QTL mapped on 1st chromosome

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Abstract
A total of 16 common rice lines and one wild rice relative grown in rarh south Bengal were genotyped using six polymorphic microsatellite markers associated with *saltol QTL* mapped on rice chromosome 1. DNA fingerprint profiles identified each of the 17 rice genotypes unequivocally and the pair-wise polymorphism data for the studied genotypes were used to analyze the genetic diversity present within the studied rice lines. The number of alleles per SSLP marker, the size range of the PCR products and the polymorphism information content (PIC) values of each marker were calculated out. A dendrogram was constructed using the average linkage (within group) and similarity coefficient among the studied genotypes which indicated that a considerable amount of genetic diversity is present and classify the studied genotypes into two major clusters and one minor cluster.

Keywords: DNA fingerprint, SSLP Markers, Rice microsatellites, QTL

The rate of increase of rice production in this decade has been slowed down in comparison to previous decade largely due to effect of different abiotic stresses, which are prevalent to rice fields of most of the rice growing regions of West Bengal. Out of different abiotic stresses salt stress have a great detrimental effect next to drought. The rice growing regions of South Bengal include a range of diverse agro ecological niches with a number of diverse rice lines. Though once (before 1970's) all the rice fields of Bengal were occupied by a number traditional (folk) lines, in the post green revolution decade a good number of such lines were replaces by high yielding rice lines. The very few existing traditional rice lines of Bengal which are still maintain by few growers in some restricted pockets has been reported to be tolerant against a number of abiotic stresses. The genetic relationship among the different diverse lines presently grown in South Bengal has been assessed by number of workers (Dey *et al*., 2005., Chattopadhyay *et al*. 2008). Assessment of genetic diversity is very important in rice breeding as it provides the basis for selection, conservation and proper utilisation (Mohammadi-Nejad *et al*., 2008; 2010). Genetic diversity is commonly measured by calculating the genetic distances among the different diverse rice genotypes (Nei and Li, 1979). The one way to assess the genetic distance is molecular profiling using a number of molecular markers (DNA fingerprinting) which reveal the differences among rice accession at DNA level and thus provide a more relevant and efficient tool for germplasm characterisation and bar-coding of different varieties for proper identification (Lang *et al*., 2000; 2001., Rahman., 2010). In rice, microsatellites are abundant and well distributed throughout the genome (Akagi et al., 1996; McCouch et al., 1997). They are valuable as genetic markers because they are codominant, detect high levels of allelic diversity, and are assayed efficiently by the Polymerase Chain Reaction (McCouch et al., 1997; Temnykh et al., 2000). Molecular proofing using different markers system is very much significant in present scenario of plant variety protection and issues related to intellectual property rights (IPR). In this present work 16 diverse commonly grown rice genotypes and one wild rice relative grown in rice field of South Bengal were genotyped using six polymorphic SSR markers linked to *saltol* QTL located on 1st chromosome of rice as reported by Gregorio *et al*., 1997; Bonilla *et al*., 2002. This QTL governed the Na+ / K+ uptake ratio and accounted for 64.3 to 80.2% of the phenotypic variation in salt tolerance (Mohammadi-Nejad *et al*., 2010).

Materials and Method

Plant materials:

A total of 17 rice genotypes were selected for the present work which included 8 West Bengal traditional rice lines, 2 exotic lines, 6 improved rice lines and one wild rice relative (*Oryza rufipogon*). The details of the different genotypes used in this experiment are given in the table 1. The different rice lines were collected from different localities of south Bengal.

Isolation of genomic DNA:

Rice genomic DNA was isolated by a standardized protocol (Dey *et al*., 2005) with some modification as below:

For all of the rice lines DNA was isolated from 6 days old seed lings whereas for *Oryza rufipogon* dried leaf

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Table 1: - The rice genotypes used in this study

Ac no.	Name	Pedigree/History
VB1	Pokkali	Typical salt tolerant traditional lines grown in coastal region
VB2	IR72046	High yielding drought sensitive salt tolerant lines used in international trial
VB3	Seeta Bhog	Aromatic medium drought tolerant folk lines
VB4	Gobinda Bhog	Aromatic medium drought tolerant folk lines
VB5	Danaguri	Aromatic medium drought tolerant folk lines
VB6	Nona Bokra	Typical salt tolerant traditional lines grown in coastal region
VB7	IR50	High yielding drought sensitive lines used in international trial
VB8	TN1	Typical submergence tolerant short (plant type) improved line
VB9	IR36	High yielding drought sensitive lines used in international trial
VB10	CSR28	High yielding salt tolerant line
VB11	Baid Julur	Typical drought tolerant traditional lines grown in drought prone area of Bankura and Purulia
VB12	Sankar Sail	Medium drought tolerant traditional line
VB13	Pusa Basmati	Aromatic fine grained drought sensitive line
VB14	Amrapalli	Typical drought tolerant improved lines grown in drought prone area of Burdwan and Hooghly
VB15	Rup Sail	Typical drought tolerant traditional lines grown in drought prone area of Bankura and Purulia
VB 16	Gorah	Highly drought avoiding rice lines of drought prone region of Purulia, Bankura and in Jharghand
VB17	O. *rufipogon*	Wildly grown with aman rice in low land rice fields

samples were used using the following protocols:

10 seeds of each rice lines (except O. *rufipogon*) were surface sterilized with freshly prepared 0.1% (w/v) mercuric chloride for 10 min, washed separately with single distilled water, soaked in distilled water for 12 hours and incubated on moist cotton in 90mm plastic Petri dishes at 37°C in the dark for 3-5 days. The shoot portions of 10 etiolated seedlings, 1.5-2.0cm in length were excised with a pair of forceps and homogenized in 850µl of a nuclear extraction buffer containing 15% (w/v) sucrose, 50mM Tris-HCl (pH 8.0), 50mM Na_2EDTA and 250mM NaCl. Homogenization was carried out using pre-chilled (-20°C) mortar and pestle placed on ice. For O. *rufipogon* suitable amount of dried leaf sample were used as plant material. The homogenate was centrifuged at 10,000 rpm for 8min at 4°C, the supernatant decanted and the pellet suspended in 700µl of a lysis buffer containing 10mM Tris-HCl (pH 8.0) and 1mM Na_2EDTA. The nuclei were lysed by adding 200µl of a 10% (w/v) solution of Sodium Lauryl Sulphate and heating at 70°C for 15min in a water bath. The solution was gradually cooled to room temperature, 200µl of a solution of 7.5M Ammonium Acetate added, the suspension mixed thoroughly by repeated inversion, incubated on ice for 2-5hours, and centrifuged at 10,000 rpm for 10min at 4°C. To the supernatant, 800µl of pre-chilled (-20°C) isopropanol was added, incubated overnight at 4°C, and the DNA precipitated by centrifugation (10,000rpm, 10min, 4°C). The DNA pellet was washed thrice with 70% ethanol, air dried and dissolved in 80µl of TE (Tris-HCl 10mM at pH 8.0 and Na_2EDTA 1mM) buffer by placing at 4°C for 12hours. To the 80µl of dissolved DNA, 5µl of a solution containing 10µg/µl of RNase-A (Fermentus Life Sci.) was added and the mixture incubated at 50°C for 30min in water bath. The solution was brought gradually to room temperature; the volume made up to 400µl by addition of TE buffer and extracted with an equal volume (400µl) of Tris-saturated Phenol: Chloroform (1:1) solution (to remove the proteins). The mixture was centrifuged at 8,000rpm for 8min at room temperature, the aqueous phase removed and 1/10th volume of 3M Sodium Acetate solution at pH 5.2 was added. The DNA was precipitated by addition of an equal volume of pre-chilled (-20°C) isopropanol and incubation at 4°C for 12hrs. The DNA was pelleted by centrifugation at 10,000rpm for 10min, the pellet was washed thrice with 70% ethanol, air dried and dissolved in 80µlTE. The purified DNA was quantified by a UV-Spectrophotometer at 260nm, the concentration of DNA adjusted to 100ng/µl, and stored at -20°C in 25µl volumes. The quality of the isolated DNA was ascertained by 0.8% agarose gel electrophoresis and Ethidium bromide staining following Sambrook *et al.* (1989).

Quantification and optimization of DNA concentration:

The amount of genomic DNA was quantified at 260nm spectrophotometrically using the absorbance reading obtained for DNA sample of each rice variety, the original DNA concentrations were determined and adjusted to 25 ng/µl.

Table 2: - Details of SSLP markers used in this study

RM Marker	Location	Motiff	Primer sequence (F)	Primer sequence (R)
RM1287	Chr.1	(AG)17	5'GTGAAGAAAGCATGGTAAATG3'	5'CTCAGCTTGCTTGTGGTTAG3'
RM8094	Chr.1	(AT)31	5'AAGTTTGTACACATCGTATACA3'	5'CGCGACCAGTACTACTACTA3'
RM3412	Chr.1	(CT)17	5'AAAGCAGGTTTTCCTCCTCC3'	5'CCCATGTGCAATGTGTCTTC3'
RM10745	Chr.1	(TATG)9	5'TGACGAATTGACACACCGAG-TACG3'	5'ACTTCACCGTCGGCAACATGG3'
RM10772	Chr.1	(CTT)16	5'GCACACCATGCAAATCAATGC3'	5'CAGAAACCTCATCTCCACCTTCC3'
RM10764	Chr.1	(AT)28	5'AGATGTCGCCTGATCTTGCATCG3'	5'GATCGACCAGGTTGCAT-TAACAGC3'

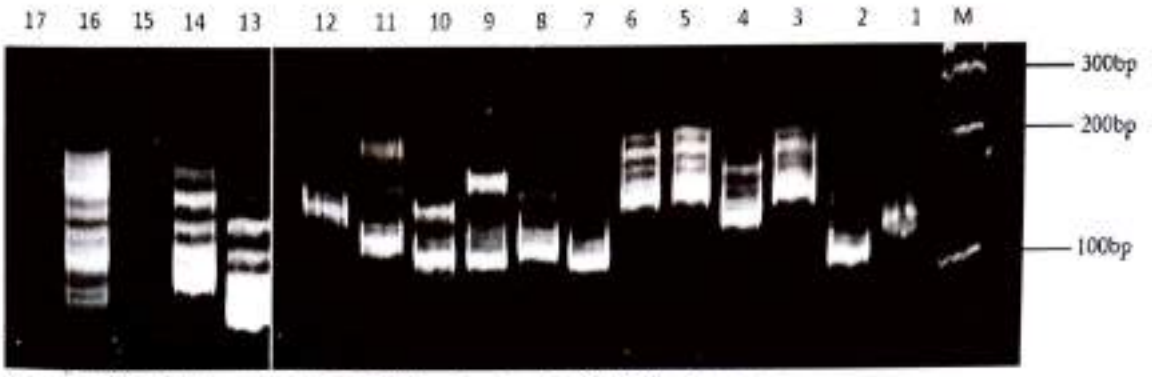

Figure-1: PAGE photograph showing PCR products using RM8094 primer pair

Identification and selection of microsatellite/SSR primers:

Six tightly linked SSR markers to the *Saltol* positioning at 10.8 to 12.28 Mb (Mohammadi-Nejad *et al.*, 2010) were selected from the available data-based search (http://www.gramene.org/) for rice SSR markers as described by Akagi *et al.* (1996), Panaud *et al.* (1996), Temnykh *et al.* (2000, 2001) and McCouch *et al.* (2002). The details of the RM primers is given in table-2.

Microsatellite/SSR analysis:

SSR primers were obtained from Integrated DNA Technologies, USA. DNA amplification was carried out in 25µl volumes using 200µl thin-walled PCR tubes (Axygen, USA) in a thermal cycler (M.J. Research). Each reaction mixture contained 1µl of genomic DNA (100ng), 0.5µl of each of the two primers (at a concentration of 10pmole/µl), 2.5µl of a 10X PCR buffer, 0.75µl of a 50mM $MgCl_2$ solution 0.25µl of a 2.5mM dNTP mixture, 0.2µl (1 unit) of a 5 unit/µl Taq DNA polymerase and 19.3µl of PCR-grade water. All the PCR reagents were obtained from Fermentas Life sci, USA. The thermal cycling conditions for the first cycle were 97°C for 5mins, 55°C for 2min. For the next 35 cycles the temperature regime was 95°C for 1min, 55°C for 1min, and 72°C for 2min. The final extension was at 72°C for 10min.

Electrophoretic separation and visualization of PCR products:

PCR was confirmed by a horizontal electrophoresis system with 2.5 % agarose (Himedia) gel. The PCR products were resolved by native polyacrylamide gel electrophoresis (PAGE) following the protocol given in Sambrook *et al.* (1989) in a 6% polyacrylamide gel.

Table 3 - Number of alleles and polymorphism information content (PIC) value of studied RM primers for 17 rice genotypes

Marker	Amplicon size (bp)	No of alleles	PIC value
RM1287	147-192	5	0.869
RM8094	166-220	6	0.943
RM3412	225-260	6	0.831
RM10745	182-201	5	0.715
RM10772	321-386	3	0.865
RM10764	131-171	2	0.114

Prior to electrophoresis each PCR-products was prepared with loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% sucrose and 1 mM EDTA). Electrophoresis was carried out in a vertical electrophoresis tank (gel size of 16cmX14cm; Biotech, India) in Tris-Borate-EDTA (TBE) buffer at 150V supplied by a power pack (Biotech, India). The gel, after electrophoresis, was stained with Ethidium Bromide (5µg in 200ml of Tris-Acetate-EDTA buffer) for 10min, washed thoroughly three times with 200ml of double distilled water per wash, and photographed using a Gel Documentation System (Alpha Innotech Corporation). The length of the amplified DNA bands (microsatellite alleles) from the different rice genotypes was determined with reference to the 100bp DNA ladder (Fermentas Life Sci. USA) included in the gel as size marker with the Molecular Analyst Software.

Scoring and analysis of microsatellite data:

Photograph of the microsatellite DNA profiles of all rice varieties against seven primers were taken for analysis of genetic divergence among studied 17 rice genotypes. The different alleles amplified form the genomic DNA of the 17 rice genotypes were identified on the basis of their size (base pairs). A 1/0 matrix for the presence and absence of all the alleles in the genotypes were produced for the six SSLP markers. A pair-wise genetic similarity coefficient matrix between all possible pairs of the rice genotypes were calculated from the 1/0 matrix using the software SPSS 10.0 according to the Nei and Li (1979) following the formula,

$S = 2N/(Ni + Nj)$,

Where S is the similarity coefficient, N is the number of common bands between the two entries (i and j), and Ni and Nj are the total number of bands for the entries i and j. A dendrogram, based on the proximity matrix, was constructed using within group linkage with the statistical software SPSS 10.0.

Polymorphism information content, or PIC value, for the SSLP markers was calculated by the simplified formula, $PICi = 1 - Si=1^{n}P^{2}_{ij}$.

Results and Discussions

The DNA fingerprint profiles of the studied genotypes were used for assessing the genetic diversity and identification of the individual genotypes. The DNA

Table 4:The pair-wise similarity matrix obtained from the SSLP-derived DNA-fingerprints among the 17 rice genotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1	0.21	0.33	0.31	0.3	0.33	0.21	0.25	0.26	0.28	0.11	0.17	0.23	0.2	0.11	0.27	0.07
2		1	0.25	0.22	0.21	0.25	0.23	0.13	0.18	0.32	0.09	0.1	0.16	0.05	0.1	0.09	0.06
3			1	0.35	0.47	0.42	0.33	0.19	0.3	0.30	0.26	0.16	0.18	0.18	0.22	0.21	0.21
4				1	0.31	0.4	0.22	0.31	0.38	0.29	0.17	0.25	0.3	0.21	0.12	0.29	0.16
5					1	0.38	0.34	0.2	0.26	0.28	0.27	0.17	0.14	0.2	0.23	0.22	0.23
6						1	0.33	0.28	0.3	0.26	0.21	0.27	0.27	0.25	0.16	0.26	0.14
7							1	0.21	0.27	0.28	0.23	0.2	0.29	0.16	0.2	0.19	0.06
8								1	0.31	0.2	0.22	0.41	0.33	0.26	0.23	0.33	0.15
9									1	0.29	0.23	0.25	0.3	0.21	0.18	0.29	0.16
10										1	0.08	0.13	0.23	0.1	0.13	0.17	0.11
11											1	0.2	0.21	0.23	0.33	0.25	0.18
12												1	0.27	0.33	0.28	0.26	0.2
13													1	0.18	0.16	0.31	0.07
14														1	0.25	0.30	0.25
15															1	0.2	0.2
16																1	0.18
17																	1

1-Pokkali, 2-IR72046, 3-Seeta Bhog, 4-Gobinda Bhog, 5-Danaguri, 6-Nona Bokra, 7-IR50, 8-TN1, 9-IR36, 10-CSR28, 11-Baid Julur, 12-Sankar Sail, 13-Pusa Basmati, 14-Amrapalli, 15-Rup Sail, 16-Gorah, 17-*O. rufipogon*

amplification profile of the 17 genotypes using RM8094 is given in figure-1. The number of alleles per SSLP marker, the size range of the amplicons and the PIC values of the markers are given in Table-3. In the present study 27 alleles were identified across 17 rice genotypes using six rice microsatellite primers. The marker RM3412 and RM8094 produced maximum (six), while RM10764 generated only two alleles across the studied genotype. The PIC value range from 0.114 to 0.943 with the average value of 0.722. The similarity coefficient (Table-3) ranges from 0.0625 to 0.476. The dendrogram (figure-2) constructed using average linkage within the group based on similarity coefficient showed two major clusters (A and B) and one minor cluster (C). Within the cluster-A, two salt tolerant lines (Pokkali and Nona Bokra), two medium drought tolerant indigenous lines (Seeta Bhog and Danaguri) and one improved high yielding line (IR-50) were included. In the cluster-B there were a number of traditional lines with high drought tolerance (Gorah, Sankar Sail, Amrapalli, Rupsail and Baid Julur), medium drought tolerant

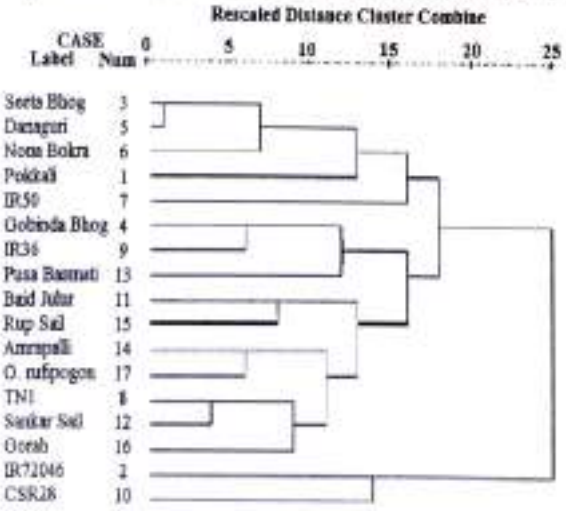

Figure-2: Dendrogram of 17 rice genotypes using average linkage (between groups)

(Gobind Bhog), high yielding drought susceptible (Pusa Basmati and TN-1) rice lines had been included. The most significant candidate of this cluster is *Oryza rifipogon* which is wild rice relatives. The third cluster (C) included two improved salt tolerant rice cultivars (IR72046 and CSR28).

The PIC value of the marker RM8094 is the highest (0.943), which amplify AT repeat whereas RM10764 is the least informative having a PIC value 0.114. This primer (RM10764) also amplify the AT repeat. From this finding it can be concluded that in the studied genotype the AT motif is more abundant within the studied genotypes. Both the marker (RM8094 with RM3412) showed maximum allele (six) which amplify AT and CT repeat respectively. RM10764 produces only two allele which amplify one AT repeat motif. The genotype CSR28 is the most diverse genotype amongst the 17 rice lines used whereas *O. rufipogon* is the least diversified rice line included in this study.

From the similarity coefficient and dendrogram it can be concluded that the Danaguri and Seeta Bhog which are indigenous aromatic folk rice line of Bengal and can tolerate medium exposure of drought are close relative of each other, whereas the *O. rufipogon* and IR-50 are distant. *O. rufipogon* is a wild relative of commonly grown rice (*O. sativa*) where as IR50 is a improved rice lines and developed and release by ICAR. Though the pokkali and Nona Bokra shared the same cluster with other genotypes there distance is 0.67. Though both are related and salt tolerance this discrepancy arise due to less number of SSLP marker linked to salTol QTL. In the same way in cluster-B a number of quite unrelated genotypes included which is also due to inclusion of very few RM primers used for genotyping. This result would be useful to know genetic variation, population structure, parentage assessment, genome mapping, Marker Assisted Selection (MAS), stock purity etc. of different rice lines. This work may also be used as the baseline information for undertaking any breeding program. As the studied genotypes is a heterogeneous

collection having salt tolerant, drought tolerant, drought susceptible and only salt tolerant RM markers were used for molecular profiling, therefore the dendrogram constructed is least informative. The investigation would be more significant and useful if the number of SSLP primers spanning all the chromosomes linked to other different stress tolerant QTLs were included in the study.

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