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Genetic profiling of a small heterogeneous population presenting traditional, wild and wild relatives of rice (*Oryza sativa* L.) in relation to osmotic stress tolerance

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Abstract
Biochemical and genetic profiling of five rice lines which included one high yielding drought and salt sensitive cultivar (IR36), one drought avoiding indigenous line (Gorah), one salt tolerant popular cultivar (Nona Bokra), one wild rice (*Oryza rufipogon*) and one wild halophytic rice relative (*Porteresia coarctata*) were done in relation to osmotic stress tolerance. Biochemical analyses were done for three compounds (proline content, total chlorophyll and total protein) which are quantitatively vary under different osmotic stresses. The rice lines were genotyped for seven osmotic stress tolerance linked rice SSR loci and DNA sequence analysis was done for the amplified product of a salt inducible gene (*salT*) using two sets of allele mining primers. The varied biochemical profiles and growth habitat of the studied rice lines were partially confirmed both by the fingerprint analysis using rice SSRs (Simple Sequence Repeats) and DNA sequence analysis of the amplified product for the selected salt inducible gene.

Key words: Rice, Osmotic stress, *Saltol* QTL, SSR fingerprint, Microsatellite panel, *SalT* gene

Land races, wild species and wild relatives of different crop genera, growing in varied agro-ecological niche, constitute a rich source of crop genetic diversity. Investigation, cultivation and preservation of those play an important role in sustainable agriculture, resulting in conservation of crop genetic resources through inhibition of monoculture (Newton *et al.* 2010). Rice like other crops have a long list of such valuable hidden genetic resources that harbour a number of favourable genes that can be exploited in rice breeding programme for development of new cultivars (Ogbu *et al.* 2010). The first step for proper utilization and popularization of these germplasm in breeding programme is the proper characterization for the different desirable agronomic traits. As all the agronomically important traits are under polygenic control, the only way to dissect those traits is development of trait linked markers and respective QTL identification (Ram *et al.* 2007, Lang and Buu 2008). Once a trait linked marker is developed, it can be used for screening of a number of genotypes in relation to the target trait. In our earlier work (Karmakar *et al.* 2012) we have profiled a total of 10 typical drought tolerant traditional upland rice lines using trait linked molecular markers. The objective of the present work was proper biochemical screening, trait linked marker based genotyping and DNA sequence analysis for a candidate gene using two different sets of allele mining primers in relation to osmotic stress tolerance for a small heterogeneous collection of rice genotypes which included commonly grown cultivars, wild species and also wild relatives.

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Materials and Methods

Plant Materials

A total of five rice genotypes were investigated, the detailed descriptions including their growth habit, distinctive morpho-taxonomic identity, specific physiological notes and place of collection are in given in Table 1.

Biochemical screening

Quantitative estimation of three biochemical compounds (proline, total chlorophyll and total protein) commonly associated with vital life processes of plants were done from 30 days old

Table 1. Detail description of the studied five rice genotypes

Our Acc. number	Name of the Genotype	Growth/habitat	Distinctive Morpho-taxonomic character	Special physiological properties	Place of collection
VB9	IR 36 (*O. sativa* L. var. IR 36)	High yielding variety, developed in IRRI, now common in all rice growing region having irrigation facility.	Grain Golden Yellow in colour with Length and Breadth ratio (L/B) 3.83, kernel white with L/B ratio 3.35.	Highly osmotic stress (drought and salinity) susceptible.	Bose Institute farm, Kolkata, India.
VB17	Gorah (*O. sativa* L. var. Gorah)	Less cultivated indigenous line grown in drought prone area of Jharhkhand and Rarh Bengal.	Grain grey in colour with L/B ratio 2.53, kernel brownish with L/B ratio 2.25.	Typical drought avoiding line with short life cycle duration (about 70 days).	Nadia, West Bengal, India.
VB18	*O. rufipogon Griffiths* (Popular name Red rice and by some tribal as Orhidhan)	Wild rice species, annual but some plants grown in small shallow water body grows as perennial.	Grain yellowish redish in colour with L/B ratio 3.94 and kernel redish in colour with L/B ratio 3.72. Grains having a typical long awn.	Grown with rice as weed, shatter readily before harvest, so that field became thoroughly infested with dropped seeds, which can grow with the next crop, remain viable for 3 years.	Jamtora, Bankura, West Bengal, India.
VB170	Nona Bokra (*O. sativa* L. var. Nona Bokra)	Traditional popular cultivar, commonly cultivated in South India and also in coastal area of Sundarban, Bay of Bengal, used as donor in salt breeding programmes.	Grain Brownish yellow in colour with L/B ratio 2.88 and kernel light brownish with L/B ratio 2.86.	Typical salt tolerant lines and Extreme late heading.	Central Saline Soil Research Institute, Canning, 24-Pargana, West Bengal, India.
VB174	*Porteresia coarctata* (Roxb.) Tateoka (Syn. name *O. coarctata* Roxb.) (local name Bunodhan)	A tetraploid wild rice relative, grows as mangrove communities along the estuaries in India.	Leaf narrow with sharp edges and spiny tip, spikelet non-conventional, grain and kernel minute, Endosperm hardy, embryo typically large with epiblast, and scuteller tail.	Highly salt and submergence tolerant, can withstand up to 36% salinity, typical perennial grass.	Estuaries, Canning located at Bay of Bengal, 24-Pargana, West Bengal, India.

fresh leaf tissue using standard protocol (Roy *et al.* 2009, Sadasivam and Manickam 2010, Karmakar *et al.* 2012 respectively).

Genetic profiling using osmotic stress tolerance linked rice SSRs

Isolation of genomic DNA

Genomic DNA of the studied rice genotypes were isolated from fresh healthy seedlings, following a pre-standardized protocol (Lodha *et al.* 2011, Roychowdhury *et al.* 2012) of our laboratory and the concentration was adjusted to 25 ng/µl.

Marker selection for genotyping

Seven salt tolerance linked rice SSR loci [Rice Microsatellite (RM)] associated with *Saltol* QTL spanning 1.4 Mb positioned at 10.9 Mb to 12.3 Mb on rice chromosome 1 of *indica* rice (Bonila *et al.* 2002, Niones 2004, IRGSP 2005, Mohammadi-Nejad *et al.* 2008, 2010) and mapped between 11.1Mb to 14.6 Mb in a japonica rice (Haq *et al.* 2010, Thomson *et al.* 2010) were selected for genotyping of the studied lines. A freely available comparative web data resource of cereal crops (Gramene database, www.gramene.org) was used for collection of detailed information of these markers. The selected primers were synthesized from Integrated DNA Technologies (IDT, USA), the details of selected SSR markers are given in Table 2.

PCR amplification

PCR amplification was done in 25 µl of reaction mixture containing 4 µl of genomic DNA (100 ng), 2.5 µl of 10X Taq-buffer, 1.0 µl of 50 mM $MgCl_2$, 0.25 µl of 2.5 mM dNTPs, 1.0 µl of each of the

Table 2. Details of the used rice SSR and gene specific allele mining primers

SSR	Ann. Temp. (°C)	Repeat Motif	Nucleotide sequence	
			Forward Primer (5′-3′)	Reverse primer (5′-3′)
RM1287	55	$(AG)_{17}$	GGAAGCATCATGCAATAGCC	GGCCGTAGTTTTGCTACTGC
RM8094	51	$(AT)_{31}$	AAGTTTGTACACATCGTATACA	CGCGACCAG TACTACTACTA
RM3412	55	$(CT)_{17}$	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
RM10745	59	$(TATG)_9$	TGACGAATTGACACACCGAGTACG	ACTTCACCGTCGGCAACATGG
RM10764	58	$(AT)_{28}$	AGATGTCGCCTGATCTTGCATCG	GATCGACCAGGTTGCATTAACAGC
RM493	56	$(CTT)_9$	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
RM140	61	$(CT)_{12}$	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG
SalT gene (Z25811)				
Pr-5 (*SalT*5'-3')	55	--------	CCACGAAGACTATGACGCTGGTG	CTTTGACCACTGGGAATCAAGG
Pr-6 (*SalT* NC)	55	--------	ATGACGCTGGTGAAGATTGGCC	GGTGGACGTAGATGCCAATTGC

forward and reverse primer (at a concentration of 10 pmole/µl) and 0.1 µl (5 U/µl) Taq-DNA polymerase. The PCR profile starts with 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min with a final extension at 72°C for 7 min using a thermal cycler (M. J. Research, MC 013130). All the PCR reagents were purchased from Fermentas Life Sciences, USA.

Polymorphism screening, scoring and analysis of the amplified products

The amplified products were resolved through 6% native polyacrylamide gel and documented using a gel documentation system (Perkin Elmer, Geliance 200 imaging system). The molecular weight of the different amplified products (allelic variants) of the SSR markers across the studied lines was determined using analysis software (AlphaEaseFC 4.0, USA). The banding patterns obtained were scored in a binary matrix and used for cluster analysis considering the statistical parameter (complete linkage between and Euclidean distance among the groups) using the statistical software SPSS 10.0. For find out the polymorphism detecting ability of the used RM markers across the studied lines polymorphism information content (PIC) value of each marker was calculated following a formula ($PIC_i = 1 - \text{“}P^2_{ij}$ where i = 1 to n and P_{ij} represents the frequency of j^{th} allele for the i^{th} marker (Liu and Muse, 2005).

Allele mining for salt inducible tolerance gene

For allele mining of salt-inducible rice gene (*salT*, Z25811) two different sets of primers were used as described by Latha *et al.* (2004). The first one was the 5′-3′ primer selected from 5′ and 3′ untranslated region immediately outside the coding region, for amplifying the total coding portion along with introns. The second was the NC primer selected only from the coding region close to the regions encoding the N- and C-termini of the coded protein. The details of the primer sequences are given in Table 2. For PCR amplification same profile was followed as in SSR genotyping. The amplified products were resolved in 1.5% agarose gel for PCR conformation. The amplified products were purified using Quiagen gel extraction kit and sequenced from a sequencing company (Chromus Biotech, Bangalore, India) and the derived sequences were analyzed using bioinformatics tools.

Results and Discussion

Proline content (µg), chlorophyll content (mg) and total protein content (mg) quantified from fresh mature tissue for the studied rice lines are given in Table 3. The proline content estimated for the studied rice lines showed no correlation with their growth habitat, particularly to osmotic stress tolerance except for one genotype. Though IR36 is a high yielding variety and very much susceptible to drought and salinity, it showed the highest proline contents among the investigated lines. But being

Table 3. Overall biochemical estimation for the studied genotypes

Genotypes	Proline content (μg/g)	Chlorophyll content (mg/g)	Total Protein (mg/g)
VB9	72.8	0.889	36.9
VB17	61.2	1.753	41.4
VB18	43.6	0.791	79.3
VB170	52.4	1.690	55.4
VB174	58.8	1.629	48.5

highly salt tolerant, both the Nona Bokra and *P. coarctata*, showed lower proline content than IR36. Again Gorah, a drought avoiding line showed the proline content in between the IR36 and the later two genotypes. But for the rest genotypes, *O. rufipogon*, the lowest proline content can be correlated with its susceptibility to osmotic stress. Chlorophyll content was recorded to be highest in Gorah, a typical drought avoiding line, next to this are found in Nona Bokra and *P. coarctata*, both of which are typically salt tolerant line. The lowest chlorophyll content was found in IR36 and *O. rufipogon* which are not at all tolerant to any osmotic stresses. So this finding can be correlated with their stress reaction properties as well their growth behavior. Total protein content was recorded maximum for *O. rufipogon* followed by Nona Bokra, *P. coarctata*, Gorah and least in IR36. In this finding, except the first lines rest others are reflecting their growth habitat parameters. The fingerprint profile showed that two microsatellite markers (RM10764 and RM140) were monomorphic whereas rest five (RM1287, RM8094, RM3412, RM10745 and RM493) were polymorphic for the studied genotypes. Number of total alleles, amplified product size and PIC value of each SSR with the reported product size of the amplified product for reference genotype (*O. sativa* var. Nipponbare) are given in Table 4. A total of 18 different alleles were detected for the five polymorphic loci across the studied genotypes with maximum numbers (4) as recorded for

Table 4. Number of detected alleles, amplified product size, PIC value and product size in reference genotype (O *sativa* var. Nipponbare) for the studied SSR loci

SSRs	No. of alleles detected	Amplified product size (bp) range	PIC value	Product size (bp) for reference genotype
RM1287	4	150 – 226	0.72	162.00
RM8094	4	177 – 203	0.84	209.00
RM3412	4	166 – 227	0.72	211.00
RM10745	3	166 – 186	0.56	189.00
RM10764	Monomorphic	158	0.00	237.00
RM140	Monomorphic	267	0.00	261.00
RM493	3	179 – 237	0.56	211.00

Table 5. Microsatellite marker panel

Genotypes	RM 140	RM 10764	RM1287				RM8094				RM3412				RM10745			RM493		
	A	A	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	A	B	C
VB9	267	158	150						196			216					186		218	
VB17	267	158			185		178						227				186			237
VB18	267	158		170				177					227			176			218	
VB170	267	158	150							203				203			186		218	
VB174	267	158				226					166				166			179		

RM1287, RM8094 and RM3412 and minimum (3) for RM10745 and RM493. A microsatellite panel (Table 5) was constructed using the molecular weight (base pair) of different detected alleles resulted from SSR fingerprints. The amplified product size range was found to be broadest (150 bp – 226 bp) for RM1287 and narrowest (166 bp – 186 bp) for RM10745. The SSR fingerprint derived dendrogram (Fig 1) revealed that IR36 and Nona Bokra form one subcluster to which Gorah and *O. rufipogon* joined to form a cluster. To this cluster, *P. coarctata* joined individually to form the final complete cluster. The calculated PIC value for the polymorphic markers showed its range from 0.84 (for RM8094) to 0.56 (for RM493 and RM10745).

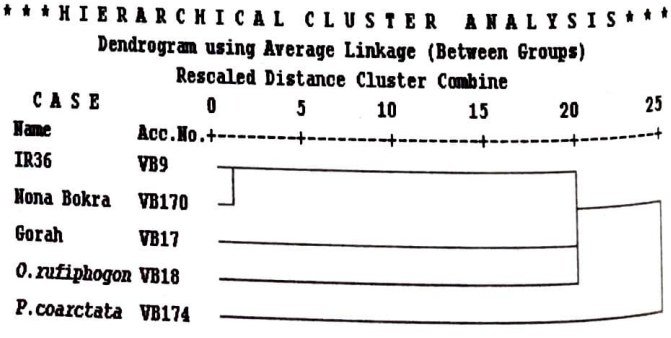

Fig 1. SSR fingerprint derived dendrogram

From the microsatellite panel it can be concluded that the five polymorphic SSR loci are highly diversified and showed about 3.6 alleles/ SSR loci across the studied five rice lines. The deviation in size of amplified product from the reference genotype (as described in Table 4) for the studied SSR loci are not so significant except for RM 10764 and RM 494. The reason for this deviation is the nature of reference genotype (Nipponbare) which is a *japonica* rice line. The PIC value for the polymorphic SSR loci is significantly high and above 0.50 which also indicating their high distinguishing ability for studied genotypes. In SSR fingerprint derived dendrogram the three true rice genotypes were grouped together to which *O. rufipogon,* the wild rice joined. *P. coarctata,* the wild rice genus became separated from the earlier cluster. Though this clustering pattern do not reflecting their growth behavior pattern, the taxonomic identity are reflected, as *O. rufipogon* is regarded as the immediate progenitor of present rice (*O. sativa*), whereas *P. coarctata* is considered as a distinct genus under the tribe *Oryzeae*. So, it can be concluded that the used SSR loci spanning the *Saltol* QTL is not sufficient for dissecting genetic diversity of this heterogeneous collection in relation to osmotic stress tolerance but have the ability to separate the studied genotypes based on their taxonomic identity. For getting more appropriate results, additional drought tolerance QTLs with linked markers should be included in addition to *Saltol* QTL. The DNA sequences obtained from the amplified products using two different primers of *salT* gene were bioinformatically analyzed and used for phylogram analysis using ClustalW2 programme which showed two different clustering patterns. The dendrogram constructed for Pr-5 (*salT* 5′-3′) (Fig 2) showed that *O. rufipogon* and

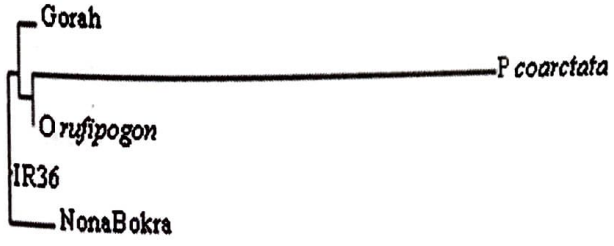

Fig. 2. DNA Sequence deried phylogram for *salT* (5'-3') primer

P. coarctata form a subcluster to which Gorah joined separately to form a cluster. The rest other genotypes (IR36, Nona Bokra) and this cluster joined separately to form the final cluster. Dendrogram derived from Pr-6 (*salT* NC) (Fig 3) showed that Gorah and Nona Bokra form one sub cluster, to which IR36 joined to form a cluster. To this cluster *P. coarctata* and *O. rufipogon* joined individually to form the final cluster. In *salT* gene sequence derived dendrogram for the Pr-5 (*salT* 5^1-3^1) primer though both *O. rufipogon* and *P. coarctata* took part in clustering, the genetic distance between the rice cluster and *P. coarctata* is much more than that of *O. rufipogon* which is again more directive to taxonomic identity than the physiological parameters. This dendrogram showed common clustering pattern with the SSR derived dendrogram.

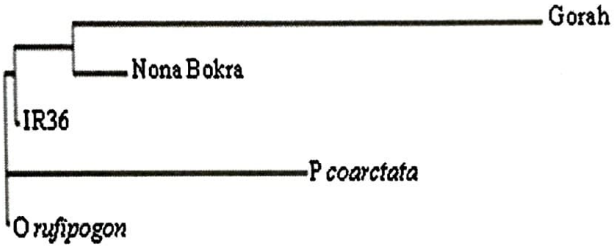

Fig. 3. DNA Sequence deried phylogram for *salT* (NC) primer

The inconclusive results derived from biochemical analysis may be due to use of normal tissue only. If stressed leaf tissues were considered in addition to the normal tissue, it may give a conclusive result. There are no earlier reports on these studied lines for the studied molecular markers spanning the *Saltol* QTLs in relation to osmotic stress tolerance and this may be the first one. The apparent lack of similarity among the clusters derived from different marker system may be due to consideration of less number of markers and respective QTLs. From sequence analysis it can be concluded that only one gene with its two sets of primers are not sufficient for sequence based profiling. Some additional osmotic stress induced genes (like *DREB, LEA*) should be considered for getting significant findings in addition to *salT* gene. Though this present study as a whole do not giving much more conclusive results, but it can be considered as a

preliminary study indicating the relationship among rice, wild rice and rice relatives in relation to salt tolerance.

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