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Identification of RAPD Markers for Salt Tolerance in Rice

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ABSTRACT. *Seventy-two randomly selected rice varieties were initially screened for salt tolerance in the greenhouse using a salt solution of 12 dS m^{-1}. They were identified into 3 major groups as salt tolerant (22 varieties), moderately salt tolerant (21 varieties) and sensitive (29 varieties). Three groups of rice varieties differing in salt tolerance were initially screened for DNA polymorphism using 60 RAPD primers. Of these, 10 primers were selected and they were used to screen all 72 rice varieties. The primers OPS 3 and OPAK 10 produced specific fragments at 510 bp and 470 bp, respectively. The fragment $OPS3_{510}$ was present very distinctly and prominently with high intensity in 17 out of 22 salt tolerant varieties. This band was either totally absent in many or faintly seen in a few moderately tolerant and sensitive varieties. $OPAK10_{470}$ was present only in 4 accessions: three salt tolerant and one moderately tolerant. Both fragments were present in proven salt tolerant varieties and absent in known salt susceptible varieties. Dendrogram constructed using the banding pattern generated by $OPS3_{510}$ grouped the accessions into the three distinct clusters of salt tolerant, moderately tolerant and sensitive varieties. The cluster analysis indicated the diversity among the varieties and helped to identify rice genotypes with wide genetic distance for future use in breeding programs and to map salt tolerant genes in rice. Because of $OPS3_{510}$ co-segregates with the salt tolerant genotypes, it could be used to identify the salt tolerant genotypes in the segregating population.*

INTRODUCTION

Rice is considered a model crop in plant biology, mainly in genetics, largely due to its compact genome (430 Mb) (USDA-RRP, 2001). Approximately 13% of the irrigated area in Sri Lanka is affected by salinity and saline soils are about 0.7 million ha. Salinity is generally associated with coastal areas and lower slope positions in the dry zone. The Mahaweli area (Zone H) is identified to contain soils with the potential to become saline (Thiruchelvam and Pathmarajah, 2000).

Rice breeding programs for salt tolerances have made use of the inherent variability found among various varieties of rice. Selection in breeding in most cases is based on various morphological and physiological parameters such as germination percentages and survival of seedling (Khan *et al.*, 1997), Na:K ratio, tissue tolerance to NaCl (Heenan *et al.*, 1988) and proline content (Igarashi and Yoshiba, 2002; Safeena *et al.*, 2002). A single marker was not able to be used for various factors, such as environmental interaction and polygenic nature of the trait (Akbar *et al.*, 1985) and considerable genetic variation observed in salinity tolerance among rice varieties (Gregorio and Senadhira, 1993). Thus, some varieties released to the saline area are not performing the expected results even though they are considered to be salt tolerant (Erickson *et al.*, 1995).

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The expression of molecular markers is stable regardless of the environment that the breeding lines are grown in. Thus, a strong association of a molecular marker with a trait will allow the breeder to more effectively select for the trait regardless of the environmental conditions. Molecular marker for *'Saltol'* gene in rice has been identified using Restriction Fragment Length Polymorphism (RFLP) (Bonilla *et al.*, 2002). Though RFLP markers are robust, the procedure involves hazardous radioactive chemicals and is tedious. Identification of PCR based non-radioactive markers will improve the efficacy of marker-assisted selection (MAS) for salt tolerance. RAPDs also detect DNA polymorphism similar to RFLP. In addition, RAPDs require less quantity of template DNA, suitable for automation, use non-radioactive materials and facilitate rapid genotyping of large germplasm (Weising *et al.*, 1995). RAPD's have been used for various applications in rice, some of which include classification of varieties (Mackill, 1995) and identification of disease resistant loci (Jenal *et al.*, 2000).

However, information relating to RAPD markers linked to salt tolerance is scanty. In this study, RAPD technology was used to identify and to establish putative molecular markers for salt tolerance in rice varieties.

MATERIALS AND METHODS

Plant material

Seeds of seventy-two rice varieties (Appendix 1) were obtained from Plant Genetic Resource Center (PGRC), Peradeniya and Rice Research Institute, Batalagoda. About a half of seeds were used for screening of rice varieties for salt tolerance and the balance was used for raising plants for DNA extraction.

Physiological screening of rice varieties for salt tolerance

All rice varieties were grown under green house conditions in hydroponics using a nutrient solution (Yoshida *et al.*, 1976). After 17 days, the seedlings were subjected to salinization (EC= 12 dS m^{-1}) by adding a mixture of NaCl (4.12 g l^{-1}), $CaCl_2$ (0.22 g l^{-1}), KCl (0.15 g l^{-1}), and $MgSO_4$ (2.24 g l^{-1}) in to the nutrient medium. None salinized nutrient medium (EC=1.0–1.03 dS m^{-1}) was used as the control. Conductivity and pH of growth medium (5–5.5) were checked and adjusted every two days. The nutrient solution was renewed once a week. The temperature of environment varied from 28-31 °C during day and 24-28 °C in the night. The experiment was conducted in a complete randomized design (CRD) with three replications. Each experimental unit consisted of 10 plants. Plants were evaluated for salt tolerance on 23rd day after salinization and morphological changes were recorded to calculate the damage index (DI) for each variety, using the IRRI criteria for screening rice varieties for salt tolerance (Yan and Tan, 1991). Statistical analysis was carried out using Duncan's multiple range test and varieties were ranked as salt tolerant, moderately tolerant or salt susceptible.

DNA extraction and assessment

DNA was extracted based on cetytrimethylammonium bromide (CTAB) method (Weising *et al*, 1995) with modification. Three grams of fresh, tender leaves from the rice varieties, which were marked as salt tolerant, moderately tolerant or salt susceptible in the above screening method, were ground in a cold mortar in liquid

nitrogen to form a fine powder. The powder was transferred to a sterile tube containing 15 ml of preheated (60 °C for 10 – 15 min) 2 X CTAB extraction buffer (1000 ml of buffer contained: 20 g of CTAB, 280 ml of 5 M NaCl solution, 40 ml of 0.5 M EDTA, 100 ml of 1 M Tris HCl, adjusted with 580 ml of deionozed water and pH was adjusted to 8.0). The clumps, if any, were broken with a glass rod and the contents were incubated for 30 min at 60 °C in a shaking water bath. An equal quantity (15 ml) of chloroform-isoamyl alcohol (24:1) was added to the tubes and they were rotated on a rotary shaker for 10 min. The extracts were collected into glass tubes after centrifugation at 5000 rpm for 10 min. The step was repeated 2-3 times until a clear solution appeared. The DNA was precipitated with ice-cold isopropanol. The DNA pellet was washed with 10 mM ammonium acetate and 70% ethanol solution and allowed to dry and resuspended in 200 µl of TE buffer. DNA concentration was estimated using a spectrophotometer (UV 1600 – UV Visible, SHIMADZU). Total DNA was diluted with sterile distilled water to a concentration of 30 ng μl^{-1} in working samples.

DNA amplification

All PCRs were performed using a Perkin – Elmer 9600 thermocycler. A series of optimization experiments was conducted where concentration of template DNA (0, 10, 20, 30, 40 and 50 ng), of primers (0, 5, 6, 7, 8, 9 and10 pMoles), of $MgCl_2$ (0, 1.5, 2.5 and 3.5 mM) and different types of Taq DNA polymerases (Taq DNA Pol., Helena; Thermoprime plus, AB Gene; Taq DNA polymerase, AB Gene) were varied to determine the optimal conditions. The optimized reaction mixture contained 10 X PCR buffer 1.5 µl, 25 mM $MgCl_2$ 1.5 µl, 10 mM dNTPs mixture (containing equal volumes of dTTP, dCTP, dATP and dGTP) 1.5 µl, Taq DNA polymerase (Thermoprime plus, ABgene) 0.13 µl, 6 pM primer 2.00 µl, Template DNA (30 ng μl^{-1}) 4 µl. The total volume of the mixture was brought to 15 µl by adding sterile distilled water. The DNA was amplified using the following temperature profile: Initial denaturation at 94 °C for 3 min followed by 40 cycles of 1 min Denaturation at 93 °C, 3 min Annealing at 35 °C, 2 min Extension at 72 °C and 10 min Final extension at, 72 °C. After thermal cycling, 3 µl of loading buffer (50% glycerol, 5 mM EDTA, 0.25% v/v bromophenol blue) was added to each sample. Amplification products (7 µl was loaded per well) were electrophoresed on 1.5% agarose gels (containing 0.5 µg ml^{-1} ethidium bromide) at 120V for 3 hrs in 1 x TAE (Tris Acetic acid EDTA). Gels were scanned under UV and photo documented using UV-photo-print gel system (Bioimage, Gel print 2000i/ VGA, USA).

Primer screening

The primers used were 10 bp oligonucliotides of random sequence from (Operon Technologies Inc, USA). A total of 60 primers (OPS 01 to 20, OPAK 01 to 20, OPN 02 to 18, OPE 01 and 02 and OPF 01) were screened in this study. Each primer was initially screened using bulked DNA samples, representing the DNA of salt tolerant, moderately tolerant and susceptible varieties. The reactions were performed at least two times to confirm the reproducibility of the amplified DNA fragments of the 60 primers initially screened, ten were selected as potentially useful for the assessment of genetic diversity and establishing putative markers for salt tolerance.

Cluster analysis

The banding patterns were scored for RAPD primer OPS3 in each rice variety. Of the 10 primers, only OPS 3 was used since it gave the most correlated results with respect to salt tolerance. Presence and absence of each scorable and reproducible band was scored as 1 and 0, respectively. A dendorgram was constructed using the binary data matrix following Unweighted Pair Group Method with Arithmetic mean (UPGMA). The data were analyzed by SAS computer package (Version 8.1).

RESULTS AND DISCUSSION

Physiological screening of rice

All varieties grew normally on the non-salinized nutrient medium (EC= 1.0-1.03 dS m^{-1}), but were affected when salt mixture was added. Although rice growth was generally inhibited by salt, marked differences in salt tolerance were observed (Table 1). They were identified into 3 major groups as 22 salt tolerant (IR 65195, PSBRC 50, Nona Bokra, At 401, BW 451, At 354, Pokkali, Jhona 349, IR 4630, BW 351, IR 51500, Kombila, BW 302, Kharamana, IR 1721, Matarawee, Moddikarruppan, Pokkalian Baticoloa, OB 678, SR 26B, Lankasamurdi and Uvarkarruppan), 21 moderately salt tolerance (Dahanala, At 353, BW 400, At 69-2, Sulai 301, At 16, IR 10206, IR 50, Podiwee, IR 9884, IR 4595, Sinnakarruppan, Suduruhondarawala, Peta, Ponni, Pachaiperumaal, Kirimurunka, IR 1702, BG 400-1, At 85-2 and Chinnapodian) and 29 sensitive (Jaya, Hartial, LIPL-01-03, *O.rufipogon*, CR 333-10, Kaluheenati, TE TEP, Suwandal, BG 401-1, BG 94-1, BG 94-1 (R), IR 28, Mawee, TN 1, IR 10198, MI 48, Basmati 217, Suduruwee, Sudumadael, Mutusamba, IR 36, BG 11-11, Paravai illankayan, LD 183-07, H 501, Sudurusamba, Nivudusuduwee, Beathheenati and Rathuheenati) varieties.

PSBRC 50 and Nona Bokra were the most salt tolerant varieties, comparable with well-known salt tolerant Pokkali. Many Ambalanthota (At) varieties were found moderately salt tolerant along with some traditional varieties like Dhahanala and Sinnakarruppan. Majority of the Bathelegoda (Bg) and traditional varieties showed salt sensitiveness. However, the validity of the results must be verified under field conditions, since the present experiment was limited to greenhouse culture.

Optimization of RAPD protocol

Large changes in concentrations (*i.e.* in order of magnitude) of template DNA did affect the amplification. The best results in terms of separation and resolution of bands were obtained with 30 ng μl^{-1} working solution. Primer concentrations affected the band number and reproducibility. With the increase in primer concentration, the number of bands either increased or appeared as smear. But at lower concentrations fragments were faint, while higher concentrations generated unseparated, large sized band, which could have been because of mispriming and misincorporation of nucleotides.

Table 1. Damage index of varieties in response to salinity.

Group	Damage index (%)	Standard Damage Index %	Variety
1 (salt tolerant)	69.8 a	60-70	IR 65195, PSBRC 50, Nona Bokra, At 401, BW 451, At 354
(salt tolerant)	70.4 a		Pokkali, Jhona 349, IR 4630, BW 351, IR 51500, Kombila, BW 302, Kharamana, IR 1721,Matarawee, Moddikarruppan, Pokkalian Baticoloa, OB 678, SR 26B, Lankasamurdi, Uvarkarruppan
2 (moderately salt tolerant)	80.6 ab	75-85	Dahanala, At 353, BW 400, At 69-2, Sulai 301, At 16, IR 10206, IR 50, Podiwee, IR 9884,IR4595, Sinnakarruppan
(moderately salt tolerant)	85.2 ab		Suduruhondarawala, Peta, Ponni, Pachaiperumaal, Kirimurunka, IR 1702, BG 400-1, At 85-2, Chinnapodian
3 (salt sensitive)	94.7 b	90-100	Jaya, Hartial, LIPL-01-03, *O.rufipogon*, CR 333-10, Kaluheenati,TETEP, Suwandal, BG 401-1
(salt sensitive)	100b		BG 94-1, BG 94-1 (R), IR 28, Mawee, TN 1, IR 10198, MI 48, Basmati 217, Suduruwee, Sudumadael, Mutusamba, IR 36, BG 11-11, Paravai illankayan, LD 183-07,H 501, Sudurusamba, Nivudusuduwee, Beathheenati, Rathuheenati

Means followed by the same letter are not significantly different, Duncan's test, P=0.05

Since dNTPs bind to magnesium ions, the $MgCl_2$ concentration required for maximum activation of Taq polymerase should be considered. At low $MgCl_2$ concentration, the number of DNA fragments amplified with a given primer either decreased or absent. However, the number of fragments amplified was directly

correlated with the increase in magnesium ion concentration to certain extent (>3 mM). Different taq polymerases gave rise to different RAPD products. Out of 3 taq polymerases used Thermoprime plus gave fairly high number of fragments with high polymorphism. The sensitivity of RAPD to changes in experimental parameters is well known (Weising *et al.*, 1995). It was found that the condition explained in the section of materials and methods of this study produced the best amplification of rice.

Reproducibility of RAPD experiments

Although most of the RAPD polymorphism which were obtained using the "optimal reaction conditions" were observed to be fairly consistent, the majority of the PCR reactions were replicated twice, or in some cases three times to confirm the reproducibility of RAPD bands and to resolve inconsistent amplification profiles. A set of samples sometimes reduced or increased slightly all band intensities with different PCRs. However, most of the bands were reproducible except for those with higher molecular weight than 3 kbp, which were mostly minor bands and varied in intensities by amplifications. Thus, those visibly reproducible bands smaller than 3 kbp were counted in the study.

Primers and polymorphism detection

Preliminary screening facilitated the selection of primers producing a higher level of polymorphism and more reproducible fragments. Such screening is essential to save time, cost and to reject primers not informative for the analysis. Once identified, the selected primers could also serve to evaluate a large number of rice genotypes in future analysis. The number of amplified fragments per primer varied from 9-17 with an average of 13.2 and the size ranged from 200 bp to 3 Kbp (Table 2). A total of 99 (75%) of these fragments behave as polymorphic bands.

RAPD markers for salt tolerance

Varieties proved to be salt tolerant, moderately tolerant and sensitive in the physiological screening were used in RAPD analysis. Out of 10 primers selected, the primers OPS3 and OPAK10 consistently produced distinct polymorphic bands. These primers were further used in screening of rice varieties to detect the RAPD fragments co-segregating with salt tolerance.

A polymorphic band unique to the salt tolerance was produced by the primer OPS 3 (Plate 1, lanes 1-6). The particular fragment at 510 bp ($OPS3_{510}$) was amplified at high intensity in 17 salt tolerant varieties (IR 65195, PSBRC 50, Nona Bokra, At 401, BW 451, At 354, Pokkali, Jhona 349, IR 4630, BW 351, IR 51500, BW 302, IR 1721, Pokkalian Baticoloa, OB 678, SR 26B and Lankasamurdi). Interestingly, this higher intensity band was either totally absent in many or very faintly seen in a few moderately tolerant and sensitive varieties. Ironically it was not seen in 5 salt tolerant varieties (Uvarkarruppan, Kombila, Kharamana, Matarawee and Moddikarruppan).

In addition, OPAK 10 produced a polymorphic band with high intensity ($OPAK10_{470}$) in 3 salt tolerant (PSBRC 50, lane 1; At 354, lane 5 and Pokkalian Batticoloa, lane 6) and one moderately tolerant (IR 4595-4-1-13, lane 8) varieties and it was absent in all others (Plate 2). Absence of bands may be due to several reasons such as deletion, duplication, inversion, transversion and point mutation *etc.* (Barua *et al.*, 1993). Both fragments identified in response to salt stress were present in varieties

proven to be salt tolerant (Pokkali, Nona Bokra, PSBRC 50 and At 354) and absent in known salt sensitive varieties (IR 28, IR 36 and BG 94-1).

Table2. Primers used and number of different amplified fragments observed among 72 rice varieties.

Primer	Sequence 5' – 3'	Total No. of bands	No. of polymorphic bands	Size range of DNA fragments (Kb)
OPAK 18	ACCCGGAAAC	15	12	2.5-0.3
OPAK 10	CAAGCGTCAC	10	8	2.4-0.3
OPAK 20	TGATGGCGTC	16	10	2.3-0.2
OPN 15	CAGCGACTGT	14	10	3.0-0.2
OPN 18	GGTGAGGTCA	9	5	3.0-0.3
OPS 10	ACCGTTCCAG	14	11	2.4-0.3
OPS 19	GAGTCAGCAG	12	9	2.9-0.3
OPS 20	TCTGGACGGA	11	10	2.7-0.2
OPS 3	CAGAGGTCCC	14	12	2.2-0.2
OPS 18	CTGGCGAACT	17	12	2.0-0.4
	Total	**132**	**99**	

Among the polymorphic fragments observed, $OPS3_{510}$ was considered to be associated with salt tolerant ability of rice. However, it could not be generalized that both fragments were undoubtedly associated with salt tolerance, since these fragments were absent in few tolerant accessions. Therefore, this needs to be verified through segregant analyses using salt tolerant and susceptible accessions selected for a specific trait for salt tolerance as parents (Gregorio *et al.*, 2002). This will be a useful approach towards marker-assisted selection (MAS) for salt tolerance improvement.

Cluster analysis was performed using the banding patterns generated by the RAPD primer OPS3. The dendrogram (Fig. 1) showed two main clusters (A and B) at 1.65 dissimilarity co-efficient. Cluster B was divided in to two sub-clusters D and E at 1.62 dissimilarity co-efficient. Cluster D consists of 17 salt tolerant varieties. Cluster A consists of all moderately salt tolerant varieties along with 5 salt tolerant varieties that did no show a band at $OPS3_{510}$. However, these 5 varieties had only 29% homology with the rest of A entries. Cluster E entirely contained salt susceptible varieties.

The dendrogram revealed that the salt tolerance varieties are diverse in reaction, coming under different clusters. RAPD marker $OPS\ 3_{510}$ may serve as a strong putative, which could be helpful in identifying the salt tolerant rice varieties. It is obvious that there is clear difference between results of the physiological screening and molecular screening of rice since the number of rice varieties falling into different groups in physiological screening was not similar to that of clusters in dendrogram. This is due to the inclusion of all the bands, generated with OPS 3. Many of these bands may be associated with other traits of the rice plant along with OPS_{510}, which is specifically identified as salinity related band. It is well known that RAPDs markers are easy, relevant and cost effective markers for screening rice varieties for salt tolerance (Erickson *et al.*, 1995). Hence the RAPD marker $OPS3_{510}$ that co-segregates with the

salt tolerant genotypes which could be used to identify the salt tolerant genotypes in the segregating population, especially in breeding programms in Sri Lanka where facilities for investigating other molecular markers are limiting.

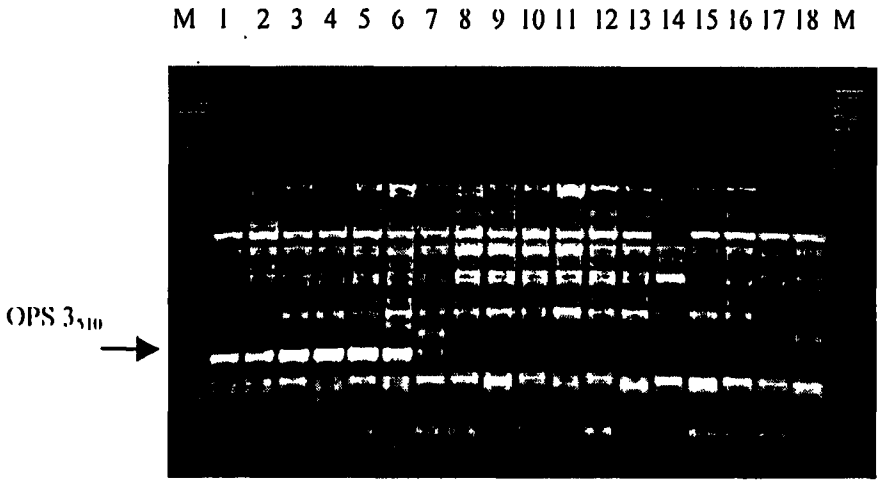

Plate 1. **Polymorphism detected with RAPD primer OPS 3.**
[Note: 1-6: salt tolerant; 7-12: moderately salt tolerant; 13-18: salt susceptible varieties] M- 1Kb ladder, 1-Nona Bokra, 2-BW 451, 3-Pokkali, 4-IR 51500, 5-BW 351, 6-At 401, 7-Dahanala, 8-At 16, 9-IR 50, 10-Sulai 301, 11-Pachaiperumal, 12-Chinnapodian, 13-BG 401-1, 14-BG 94-1, 15-IR 28, 16-Mawee, 17-TN1 and 18-MI 48

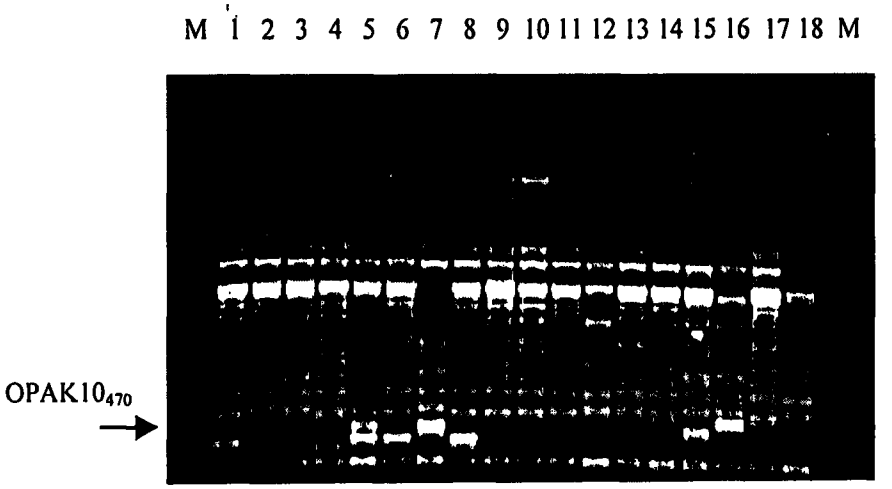

Plate 2. **Polymorphism detected with RAPD primer OPAK 10.**
[Note: 1-6: salt tolerant; 7-12: moderately salt tolerant; 13-18: salt susceptible varieties] M-1Kb ladder, 1-PSBRC 50, 2- Uvarkarruppan, 3-BW 302, 4- Lanka Samurdi, 5-At 354, 6-Pokklian Baticoloa, 7-BW 400, 8-IR4595-4-1-13, 9-At 353, 10-At 69-2, 11-BG 400-1, 12-At 85-2, 13-Mutusamba, 14-Jaya, 15-IR 36, 16-*O.rufipogan,* 17-LD 183-07 and 18-H 501

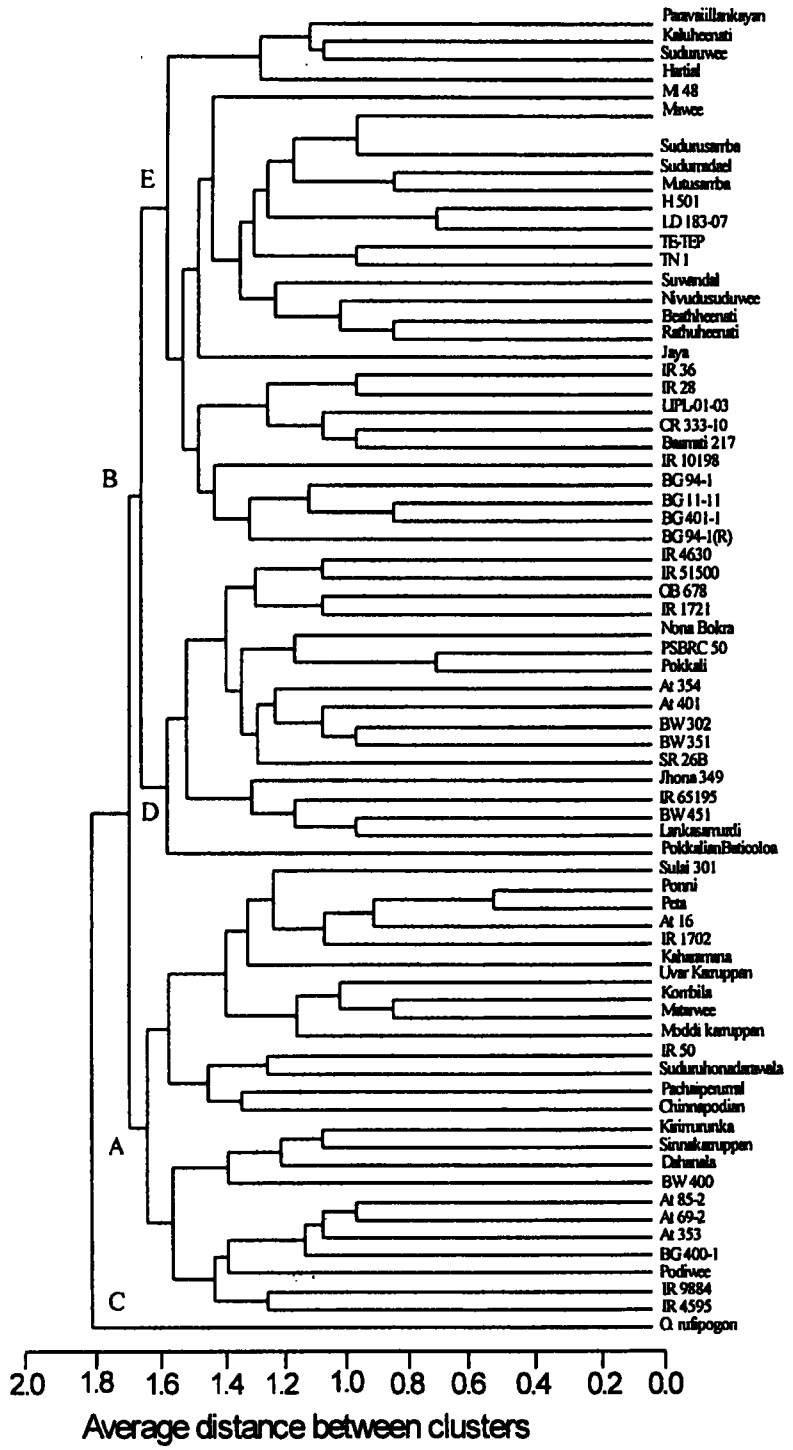

Fig. 1. A dendrogram generated by OPS 3 primer for 72 rice varieties

CONCLUSIONS

The 12 dS m^{-1}salt solution could separate all seventy-two rice varieties into three categories: salt tolerant (22 varieties), moderately salt tolerant (21 varieties) and salt susceptible (29 varieties). The modified protocol for DNA extraction yielded pure and high amounts of DNA. Among the primers used, OPS 3 is the best primer with respect to salinity screening. OPAK 10 also shows some promise. $OPS3_{510}$ appears as a very valuable putative marker linked to the salt tolerance in rice.

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APPENDIX

Appendix 1. Estimated damage index (DI %) for each rice varieties used in the study

No	Variety	DI %	No.	Variety	DI %
	IR 1702	81.11	37.	Moddi karruppan	71.59
2.	Kirrimurnka	84.02	38.	BW 451	70.34
3.	BW 400	76.64	39.	AT 401	69.00
4.	AT 16	83.00	40.	Peta	83.94
5.	Sinnakarrupan	82.64	41.	Podiwee	76.42
6.	Matarawee	68.34	42.	Suduruwee	93.47
7.	Kaharamana	70.01	43.	Ponni	86.43
8.	IR 51500	72.72	44.	Paravai illankayan	96.71
9.	Jhona 349	70.32	45.	Beath heenati	92.96
10.	IR 4630	70.53	46.	Chinnapodian	83.04
11.	Nona Bokra	60.21	47.	*O. rufipogan*	88.99
12.	Rathuheenati	92.30	48.	BG 94 - 1(R)	95.80
13.	Sudurusamba	99.61	49.	Sulai 301	80.00
14.	Suwandal	98.64	50.	CR 333 – 10	96.31
15.	IR 10206	83.16	51.	Basmati 217	98.99
16.	Mawee	96.42	52.	Jaya	92.22
17.	Kaluheenati	94.50	53.	AT 353	80.03
18.	MI 48	92.59	54.	BG 400 – 1	91.00
19.	BG 94 – 1	99.00	55.	AT 85 – 2	84.36
20.	IR 28	99.86	56.	AT 69 – 2	81.10
21.	BG 401 – 1	79.90	57.	BW 351	69.07
22.	IR 50	81.09	58.	Lankasamurdi	74.00
23.	LIPL – 01 – 03	98.76	59.	BW 302	91.86
24.	BG 11 – 11	95.97	60.	AT 354	69.03
25.	Pachaiperumal	85.89	61.	SR 26 B	76.00
26.	Suduru hondarawala	84.00	62.	Pokkalian Baticoloa	70.32
27.	TE – TEP	90.34	63.	Hartial	91.61
28.	OB 678	71.50	64.	Sudumadael	93.95
29.	Kombila	70.01	65.	Mutusamba	96.80
30.	IR 9884	76.07	66.	H 501	98.05
31.	IR 1721	68.86	67.	LD 183 – 07	97.99
32.	IR 65195	68.00	68.	Nivudu suduwee	92.78
33.	IR 4595	73.98	69.	TN 1	98.99
34.	Pokkali	65.00	70.	IR 36	97.86
35.	PSBRC 50	60.97	71.	IR 10198	95.21
36.	Uvar Karruppan	69.98	72.	Dahanala	81.03

Note: DI was calculated based on Yan and Tan (1991).