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IDENTIFICATION OF SALINITY TOLERANT ACCESSIONS OF TRADITIONAL RICE VARIETY 'POKKALI'

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ABSTRACT

A total of 36 accessions of traditional rice variety Pokkali are available at the Plant Genetic Resource Centre, Gannoruwa and at the Rice Research and Development Institute, Batalagoda. The present study was conducted to identify the real or true *Pokkali* accessions for research purposes. The accessions were morphologically characterized and physiologically screened to identify similar accession groups with salinity tolerance. The accessions were screened for tolerance to salinity at seed germination stage and at seedling stage in hydroponics using salinized (EC~12ds/m) nutrient solution. Based on tolerance salinity to ability, the accessions were grouped as Highly tolerant, Tolerant, Moderately Tolerant, Sensitive and Highly Sensitive. Selected accessions were checked to see their DNA banding pattern by using tightly linked molecular marker RM 3412. Two out of 36 *Pokkali* accessions, namely 5557 and Ac 810 were grouped in one cluster and were highly tolerant to salinity. In morphological evaluation both accessions came under one cluster and showed morphological similarity. Thus, accessions 810 and 5557 were identified as real or true *Pokkali*.

Key words. *Pokkali*, Accession, Rice, Molecular marker, Salinity tolerance.

INTRODUCTION

Rice stands as the second highest grown crop in the world (FAO, 2004). Drought, salinity and submergence are the main abiotic stresses that affect the plant growth and development of rice (Choudhary *et al*., 2009). Salinity is a major threat for agricultural production that affects ionic and osmotic as well as nutritional relation of plants and it has negative impacts on

growth rates, tillering and seed production of crop plants (Munns and Tester, 2008). Soil salinity seriously affects on crop productivity and it is one of the main soil related problems of world agriculture. FAO, (2008) has estimated that 2% of the rain-fed agriculture area (32 million ha) is affected by salinity. Salt tolerance of crop plants is known as the ability of the plant to survive and to complete its growth cycle under saline conditions (Seydi, 2003).

Ion channels are key players in maintaining ion homeostasis also under salinity. Chloride anion (Cl^-) content was very low under control conditions but at 150 mm *nacl*, Cl^- was abundantly accumulated in leaves of the salt sensitive rice line IR 29, whereas the salt tolerant line *Pokkali* excluded it from the leaves (Diédhiou, 2007). Traditional *Pokkali* is known as tall statured plant with medium bold grain of red pericarp rice. *Pokkali* has given 2 - 3 tons per hectare yield under organic farming. It possesses great medicinal properties due to the high content of iron, zinc, potassium and antioxidants (oryzanol, tocopherol and tocotrienol etc) (http://www.thehindu.com/todays-paper/tp-national/tp-kerala/pokkali-rice-farmers-bag-national-award/article3344092.ece-2017.04.21).

Among abiotic stresses, salinity stress is complex because of variation in sensitivity at various stages in the life cycle of rice plant. Salt stress not only affects the plant morphological attributes, but also disturbs the plant metabolic activities. Several strategies have been developed to reduce the impacts of salinity by using tolerant cultivars and adopting different management strategies. Tolerance to salinity varies depending on the stage of growth, adaptation characteristics of plant and species (Akbari *et al*., 2007). Several studies have indicated that rice is tolerant to salinity during germination, becomes very sensitive during early seedling stage (2-3 leaf stage), again tolerant during vegetative growth stage, becomes sensitive during pollination and fertilization and then becomes increasingly more tolerant at maturity (IRRI, 1997; Heenan *et al*., 1988; Lutts *et al*., 1995). The development of salt tolerant cultivars has been considered as one of the strategies to increase rice production under saline conditions. Screening of rice cultivars at seed germination and seedling stage is comparatively easier because only a short time required. Screening for salt tolerance in the field is difficult as soil

salinity is dynamic i.e. High variability of spatial and temporal variation of salt concentration in the soil. Growing plants in hydroponics can avoid most of such variability (David, 2004).

Last ten years, a rapid progress has been made towards the development of molecular marker technologies and their application in linkage mapping molecular dissection of the complex agronomical traits and marker-assisted breeding (Singh *et al*., 2011). Rice cultivars grown in saline soils are sensitive at both the vegetative and reproduction stages. However, salinity tolerance at different growth stages seems to be managed by independent genes. *Saltol* is a major quantitative trait locus (QTL) and was identified in the salt-tolerant cultivar Pokkali. Its location was detected on chromosome 1. This QTL confers salinity tolerance at the vegetative stage and explains about 64% to 80% of the phenotypic variation (Bonila *et al*., 2002). Several studies reported that this QTL was detected in some other rice varieties too (Ren *et al*., 2005; Takehisha *et al*., 2004). Rice microsatellite (RM) markers RM 493, RM 140, RM 8094, RM 1287 and RM 3412 were found to be linked to *Saltol* QTL on chromosome 1 (Niones, 2004) and RM 493 and RM 3412 which were found to be tightly linked to *Saltol,* can be used for foreground selection (Le *et al*., 2012). Characteristics like salt tolerance are controlled by large number of QTLs which may share homology between genes responsible for other abiotic stresses like temperature, drought, flood and submergence etc (Deeptidavla *et al*., 2013). This important traditional rice variety has been conserved at the PGRC of the department of agriculture, Sri Lanka. However there are so many accessions under the name of Pokkali so that there may be duplications. The present study was conducted to identify the real Pokkali accessions for research purposes due to many of Pokkali accessions available at PGRC.

MATERIALS AND METHODS

Plant materials

This study was carried out in *Yala* 2014 and *Maha* 2014/15 seasons at the Rice Research and development Institute (RRDI), Batalagoda situated in the Low Country Intermediate Zone. Seeds of 30 different Pokkali accessions

which are available at PGRC, Gannoruwa and six Pokkali accessions which are available at RRDI, Batalagoda were used (Table1).

Table 1. Available Pokkali accessions at PGRC, Gannoruwa and at RRDI , Batalagoda.

Available Pokkali accessions at PGRC, Gannoruwa					Available Pokkali accessions at RRDI, Batalagoda
3251	3922	5642	5561	11704	AC-809
3573	3796	5557	11699	11705	AC-810
3509	4013	5556	11700	11706	AC-811
3701	4160	5558	11701	11707	AC-812
3881	4650	5559	11702	11708	AC-813
3957	4330	5560	11703	11709	AC-445

Morphological evaluation under field condition

An experiment was conducted in *Yala* 2014 at RRDI to evaluate the morphological characteristics by using augmented design. Variety Bg 357 was used as the susceptible check and it was replicated 13 times. Plant height, Panicle number, Tip of the spikelet colour, Panicle length, Shattering percentage, Panicle weight, Seed per panicle, Empty seeds per panicle, 1000 seed weight, Grain yield, Maturity duration and Grain colour were recorded and measured at maturity stage.

Reaction to salt stress at seed germination stage

Accessions were tested for ability to sustain seed viability under high salt concentration an efficient method to screen rice for tolerance to salinity reported by Abesiriwardena (2004). Sodium chloride solutions were prepared and fifty seeds from each cultivar were soaked in the solution with 45 (dS/m) Electrical Conductivity (EC) for 9 days in Petri dishes. Seeds were taken out after soaking period and washed thoroughly with distilled water in order to remove the salt deposited around the seeds before placing them on wetted blotting paper in 9 cm diameter petridish to germinate. Seeds were allowed to germinate in 9 cm diameter petridish lined at the bottom with one piece of blotting paper. The average temperature in the laboratory during the study period was 27 ^{0}C. Number of germinated seeds was counted for each accession in each replicates after 5 days. It was a Complete Randamized

Design (CRD) with 2 replications. This experiment was repeated twice to determine the consistency of results of various accessions.

Reaction to salt stress at seedling stage

An experiment was conducted in 2014 *Yala* in the green house at RRDI. Stock solution was prepared based on the methods adopted by Yoshida *et al.* (1976). The nutrient solution was salinized by adding NaCl while stirring up to the desired EC level (3 and 6g NaCl l^{-1} nutrient solution gives an EC of 6 and 12 dS m^{-1}, respectively). Test seeds were heat-treated for 5 days in a convection oven set at 50 ^{0}C to break seed dormancy. Breaking of seed dormancy is very essential in this screening technique. After breaking dormancy, seeds were surface sterilized with fungicide and rinsed well with distilled water. Sterilized seeds were placed in petridishes with moistened filter papers and incubated at 30 ^{0}C for 48 hr to germinate. Two pre-germinated seeds per hole were sown on the styrofoam seedling float. The radicle should be inserted through the nylon mesh. The styrofoam seedling float was suspended on the tray filled with distilled water. After 3 days, when seedlings were well established, water was replaced with salinized nutrient solution. Initial salinity was at EC = 6 dS m^{-1}. Three days later, salinity level was increased up to up to EC=12 dS m^{-1} by adding NaCl to the nutrient solution. The solution was renewed in every 8 days and the pH was maintained at 5.0. Test entries were rated at 10 and 16 dS m^{-1} after initial salinization. Modified standard evaluating score was used (Table 2.) for rating the visual symptoms of salt toxicity. Experimental design was a Randomized Complete Block Design (RCBD) with three replications.

DNA Extraction

The leaf samples were cut into 2-3 cm pieces and grounded the sample with 400 µl extraction buffer by pestle and taken finally after mixing 400 µl extraction buffer into eppendorf (2ml) tube and vortex well. Eppendorf tube was placed on ice and 500 µl was added and shake on orbit shaker for 20 minutes at 600 rpm. After that it was centrifuged for 10 minutes at 14000 rpm. The supernatant was transferred into another eppiendorf (1.5 ml) tube and 900 microliters 100% ethanol or isopropanol was added and shaked gently for 30 seconds. The sample in was Kept in -20 ^{0}C freezer overnight. Then the

samples was vortexed and taken out for centrifugation at 1400 rpm for 10 minutes. The ethanol was removed and the pellets were allowed for air drying for at least one hour or more. Then 50 µl, 1 X TE buffer is added for resuspension and the sample was kept in 40^0C freezer overnight for chelating reaction. Finally the samples were vortexed and stored at -20^0C freezer.

Table 2. Modified Standard Evaluation Score (SES) of visual salt injury at seedling stage.

Score	Observation	Tolerance
100%	Normal growth, no leaf symptoms	Highly tolerant
75%	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
50%	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
25%	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
0%	Almost all plans are dead or drying	Highly susceptible

PCR Amplification of DNA

PCR reaction mixer (15 µl) included 5 µl of PCR master mix, (Promega USA Catalog No-M7502) , 5µl of nuclease- Free Water , 1 µl (20 µM) of upstream and downstream primer (As a SSR molecular marker RM 3412) and 3 µl (40ng/µl) of template DNA. Amplification reaction consists of initial denaturation for 5 min at 94 °C and of 35 cycles of 1 min at 94 °C (denaturation) 1 min at 52.1 °C (annealing) and 2 min at 72 °C (extension) followed by 7 min at 72 °C (Final extension) in a Mycycler PCR system (Bio red , USA). Amplified products were separated in 2% agarose gel (Sigma, USA), containing 0.5 ng/ml Ethidium Bromide (EtBr). Separated PCR products were visualized under UV light and photographed using Enduro™ gel documentation system.

Statistical analysis

Cluster Analysis and ANOVA

For the preparation of dendogram Grain colour, Plant height, Panicle length, 1000 seed weight and Maturity duration were considered. Cluster analysis was done using Minitab software.

Analysis of variance (ANOVA)

To check the difference among Pokkali accessions for salinity tolerance at seed germination stage and seedling stage, ANOVA was performed using General Linear Model (GLM) procedure followed by the Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSSION

Morphological evaluation under field condition

Out of 36 Pokkali accessions collected, 11 accessions were identified as white colour grain types. Grain colour, plant height, panicle length, 1000 seed weigh and maturity duration of collected Pokkali accession are presented in Table 3. Pokkali is a red rice variety, (Rebeira *et al*., 2014). According to the grain colour 25 accessions were separated out from the whole set of accessions. One of the accessions 3,701 received from PGRC showed photoperiod sensitivity. Through combining all the observations tested accessions were categorized into four groups at the -62.02 similarity level by using cluster analysis and it is presented in Figure1.

Reaction to salt stress at seed germination stage

Germination percentage of different Pokkali accessions and their mean separation are presented in Table 4. According to the mean separation four accessions showed the higher germination percentage. Accession Nos.5557, 3922, 4330, AC 810 showed more than 67.5% germination under the salinity stress. Accession nos.5560, AC 809, 3881 and all white colour grain type accessions showed less than 15% germination under salinity stress.

Reaction to salt stress at seedling stage

Salt tolerant levels of different Pokkali accessions at seedling stage are presented in Table 5. Accessions namely 5557 and Ac 810 showed higher salt stress tolerant percentage at seedling stage. Therefore, they can categorize as salinity tolerant Pokkali accessions at the seedling stage. Clustering was done for ability to sustain seed germination under high salt concentration and percentage score of salt stress tolerance at seeding stage. Five distinct clusters were identified at 79.3 similarity level and it is presented in Figure 2.

Table 3. Grain colour, plant height, panicle length, and 1000 seed weight and maturity duration of available Pokkali accessions.

Acc No.	Grain colour	Plant Height(cm)	Panicle length(cm)	1000 Seed weight(g)	Maturity duration(month)
3251	Red	114.6	29.5	26.34	4.5
3573	Red	79.0	27.1	25.54	3
3509	Red	104.4	24.7	27.42	4.5
3701	Red	100.3	24.6	22.45	4.5
3881	Red	84.7	26.9	32.14	4.5
3957	Red	106.5	27.4	28.92	4.5
3922	Red	98.0	27.7	32.9	4
3796	Red	100.7	28.7	27.84	4
4013	Red	110.3	28.7	27.78	4
4160	Red	113.5	24.3	28.12	4
4650	Red	114.0	21.1	32.34	4.5
4330	Red	107.7	27.9	36.3	4.5
5642	Red	95.9	25.8	26.86	4.5
5557	Red	108.2	26.4	29.43	4
5556	Red	90.7	25.4	27.8	3
5558	Red	101.3	30.3	28.3	4
5559	Red	110.7	27.4	22.42	4.5
5560	Red	120.2	27.9	30.16	4.5
5561	Red	116.8	29.1	25.76	4.5
11699	White	51.8	17.2	25.66	3.5
11700	White	55.1	18.1	26.36	3.5
11701	White	53.6	17.4	24.36	3.5
11702	White	50.6	16.2	27.0	3.5
11703	White	57.8	15.6	27.44	3.5
11704	White	60.1	14.8	27.14	3.5
11705	White	60.9	15.0	18.84	3.5

Cont...

Acc No.	Grain colour	Plant Height(cm)	Panicle length(cm)	1000 Seed weight(g)	Maturity duration(month)
11706	White	57.7	16.0	23.38	3.5
11707	White	55.4	14.1	28.24	3.5
11708	White	55.1	14.9	25.36	3.5
11709	White	58.2	14.8	26.74	3.5
809	Red	92.3	26.9	32.42	3
810	Red	108	25.5	29.74	4
811	Red	82.1	29.9	27.4	4.5
812	Red	82.3	31.9	33.66	4.5
813	Red	86.3	30.9	26.94	4
445	Red	85.6	28.5	22.96	3

Accession No 5557 and Ac 810 have grouped in one cluster. At seed germination stage as well as at seedling stage both Pokkali accessions showed similar level of salinity tolerance. They also showed similarity under morphological character evaluation (Table 3).

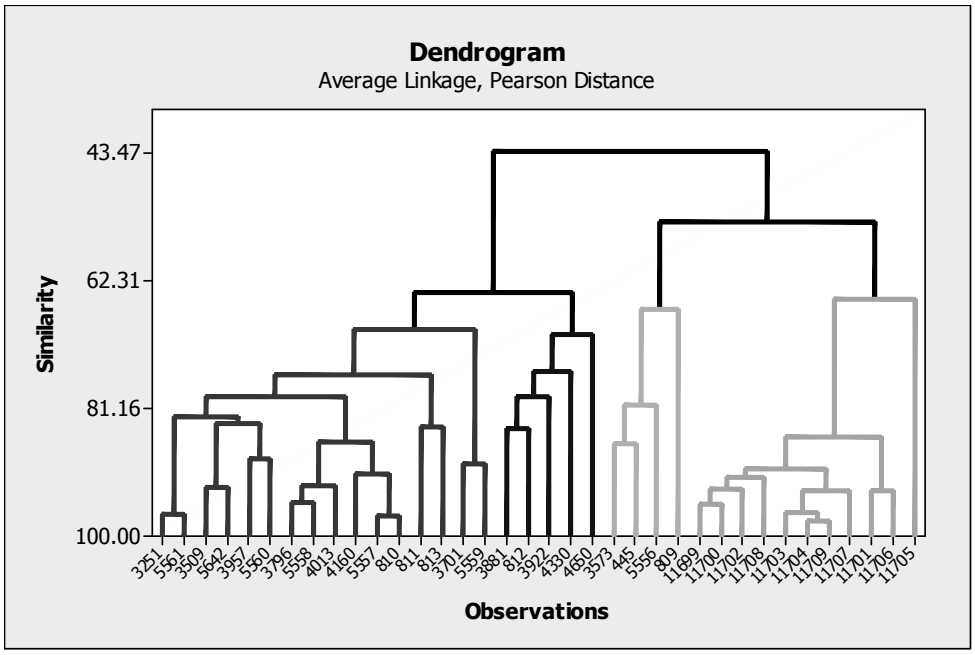

Figure 1. Dendrogram of Pokkali accessions for their Grain colour, Plant height, Panicle length, 1000 seed weight and Maturity duration.

Therefore, these two accessions can be identified as the salt tolerant real Pokkali accessions out of all available Pokkali accessions. Ac 810 is the best accession from Batalagoda accession stock and accession No. 5557 is the best accession from the PGRC accession Stock.

Polymorphism analysis

RM 3412, RM 140 and RM 493 were used as tightly linked SSR markers. Accessions with red grain color over the moderately susceptible level were used to see the banding patterns. Banding pattern of the selected accessions is illustrated in Fig 3. Accessions Ac 810 and 5,557 showed the same banding pattern with RM 3412 and RM 493. In RM 140, there was same banding pattern with Ac 810 and 5,557 and other selected accessions were appeared with polymorphic bands.

Table 4. Germination percentage and the mean separation of the different Pokkali accessions.

Acc No.	Germination percentage
3251	62 abc
3573	27.5 fghij
3509	59 abcd
3701	22 efghi
3881	5 lm
3957	62.5 ab
3922	71.5 a
3796	22.5 ghijk
4013	21 efghi
4160	59 abcd
4650	48 bcde
4330	70 a
5642	65.5 ab
5557	71.5 a
5556	43.5 cdef
5558	41.5 defg
5559	53.5 abcde
5560	11.5 klm
5561	38.5 efgh
11699	9.5 klm
11700	8 klm
11701	14 jklm
11702	1 lm
11703	0 m
11704	13.5 jklm
11705	5 lm
11706	1 lm
11707	3.5 lm
11708	6.5 klm
11709	7 klm
AC-809	8.5 klm
AC-810	67.5 a
AC-811	58.5 abcd
AC-812	35 efghi
AC-813	58.5 abcd
AC-445	21.5 efghij

Note: The mean followed by the same letter are not significantly different at p=0.05

Table 5. Salt tolerance of levels of different Pokkali accessions at the seedling stage.

Score	Accession No.	Tolerance
100%	-	Highly tolerant
75%	5557 (a), Ac 810 (a)	Tolerant
50%	3573(ab),4650(ab),11703(bc),Ac813(bc),Ac811(bc),11700(bc),11707(bc)4330(bc),5556(bc),3922(bc),5560(bc),11706(bc)	Moderately tolerant
25%	3957(bcd),812(bcd),4160(bcd),3881(bcd),3509(bcd),3796(bcd),11699(bcd),11704(bcd),Ac445(bcd),3251(bcd),4013(bcd),5558 (bcd),5561(bcd)	Susceptible
0%	5559(cd),3701(cd),5642(cd),11701(cd),11708(cd),Bg357(cd),11705(cd),11709(cd), 11702(cd)	Highly susceptible

Note: the mean followed by the same letter are not significantly different at p=0.05

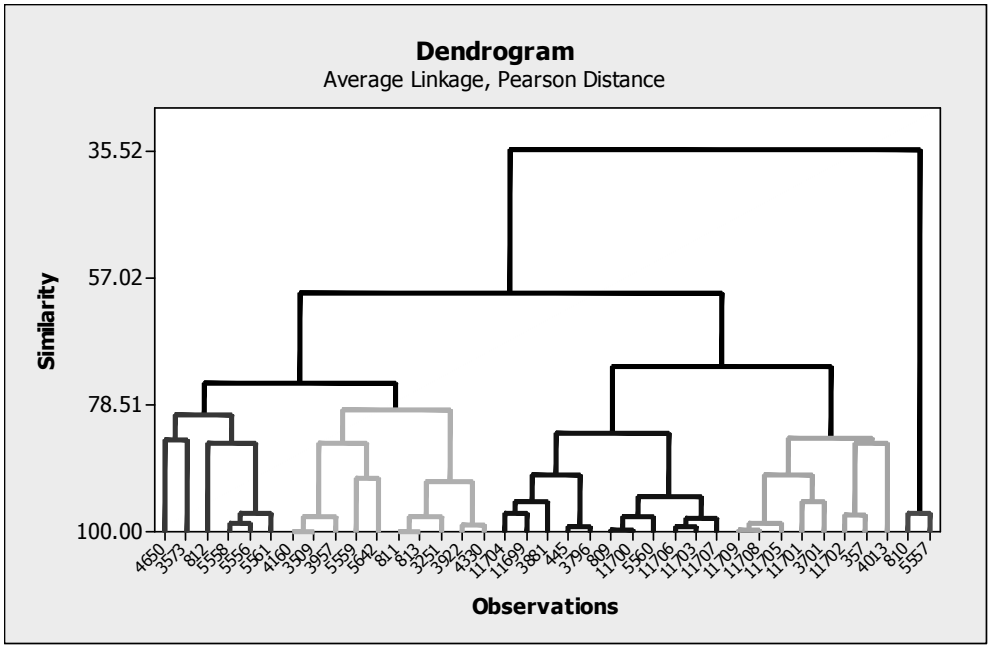

Figure 2. Dendrogram of Pokkali accessions for salt stress tolerance under seed germination stage and seedling stage.

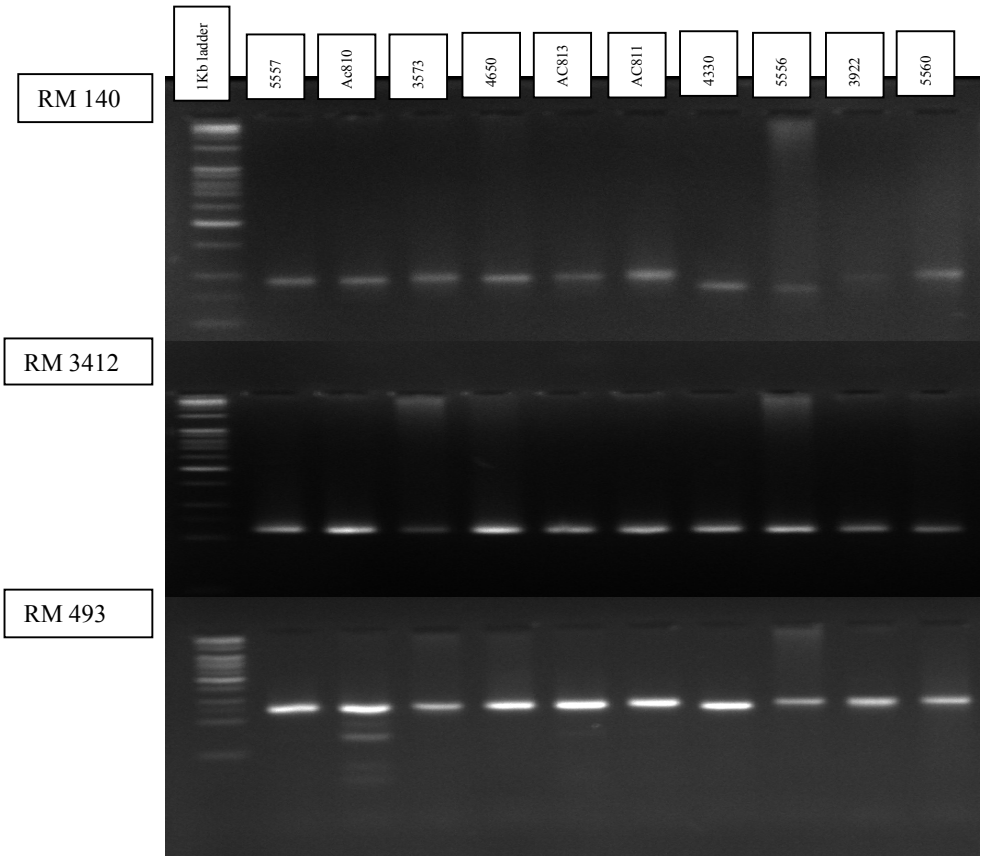

Fig 3. DNA profile of the ten selected *Pokkali* rice accessions with SSR marker RM140, RM3412 , RM 493.

CONCLUSION

Accessions Ac 810 from Batalagoda and 5557 from the PGRC are the real or true Pokkali accessions that can be used in future breeding programmes on salt tolerance of rice crop.

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