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Evaluation of Rice (*Oryza sativa* L.) Germplasm for the Identification of High Folate Accession Using HPLC

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A B S T R A C T

Folate is an essential micronutrient in human diet and its deficiency causes neural tube defects, coronary heart disease and certain forms of cancer, impaired cognitive functions. Humans depend on plant based sources for this nutrient. Rice is a major staple food but unfortunately with low folate content when compared to other plant based foods. Hence, screening and evaluation of available rice germplasm accessions for folate content is the basic step to identify folate rich accessions for further studies. In the present study, folate was extracted by a modified tri-enzyme treatment and assayed using HPLC C18 columns with a UV detector at 280 nm. The mean folate content of unpolished brown rice in 150 accessions were investigated and the results ranged from 9.721 µg/100 g to 29.284 µg/100 g revealing a three-fold difference between the lowest and highest values. Twelve lines had recorded significantly higher folate content (>20 µg/100 g), 136 lines had moderate (11 to 20 µg/100 g) and two lines had registered low level of folate (<10 µg/100 g). Cluster analysis revealed that, 150 accessions were grouped into three clusters at the similarity coefficient of 1.7. Cluster I consisted of five accessions *viz.*, RG1 (Mapillai Samba), RG2 (CK275), RG3 (Senkar), RG4 (Murugankar) and RG162 (IR 64) with high folate range of 27.263 to 29.284 µg/100 g of sample. Cluster II consisted of 12 accessions in total with two sub clusters. Cluster III also comprised of two sub clusters in which, sub cluster I had 123 accessions with moderate level of folate content (12.516 - 18.603 µg/100 g) and the remaining 10 genotypes in sub cluster II had genotypes with lower values. These results suggested that accessions from high and low folate pool can be utilized in recombination breeding to enhance the level of folate and also to map the genomic regions associated with high folate content.

Introduction

Micronutrient malnutrition also called "hidden hunger" is caused by the deficiency of micronutrients in the diet. Worldwide more than two billion people, especially women and preschool aged children are afflicts with malnutrition (White and Broadley, 2009). The major reason for micronutrient deficiency in the diet is the predominance of non-diversified cereal and plant-based diets which are poor in micronutrients as compared with the meat-rich diets (Gomez-Galera *et al.*, 2010). Moreover, processes like polishing, milling and pearling of cereals make them even poorer in micronutrients (Borg *et al.*, 2009).

Folate (Vitamin B9) occurs in foods as reduced derivatives of folic acid (pteroyl-L-glutamic acid) and is largely bound to polyglutamates. Folate nutrition is very important due to its role in the prevention of neural tube defects in the foetus and as a cofactor in one-carbon transfer reactions in both mammalian and plant cells. It is mainly involved in two important cycles such as DNA biosynthesis and the methylation cycle (Rebellie *et al.*, 2000). Folate administration decreases elevated plasma homocysteine, which is regarded as a risk factor for cardiovascular diseases. Humans lack the ability to make folate *de novo* and hence depend on plant-derived foods as the primary source (Scott *et al.*, 2000). Plant-based foods such as fruits (citrus), nuts and leafy vegetables such as spinach, lettuce, broccoli and asparagus are good source of dietary folate for humans and other animals (Rychlik *et al.*, 2016). Though the deficiency can be supplemented by capsule intake, fortification with synthetic folic acid, the natural forms of folate are better for intestinal absorption than the synthetic form. Staple foods such as wheat, maize and rice contain very low amount of folate which is insufficient to meet the recommended dietary allowances (RDA) of 400 µg per day for an adult and 600 µg per day for expectant mothers to avoid miscarriages.

Rice is a rich source of macro and micronutrients in its unmilled form. On an average, 30% of calories come from rice and this can increase to more than 70% in some low income countries (FAOSTAT, 2009). Unpolished rice is a rich source of Vitamin B complex. During polishing, majority (75 – 90%) of these vitamins is removed (USDA, 2012). Considering this, there are concerted efforts to biofortify common cereal grains with folate using transgenic approaches.

The primary step to biofortify rice can be successfully achieved after screening the folate distribution in rice germplasm accessions. The extent of natural variation in folate content needs to be determined to employ selection of genotypes or breeding for enhancement of folate levels. As such, there is very limited study in India on the genetic variation in rice germplasm for folate content. Selection of rice genotypes with higher folate content is the first step towards enhancement of folate levels in rice based foods. So the prime aim of this study was to screen a set of rice germplasm to understand the extent of genetic variation for folate content.

Materials and Methods

The genetic pool being maintained as an Association Mapping Panel at Paddy Breeding Station (PBS) of TNAU, Coimbatore formed the material for study. It comprises of 150 germplasm accessions collected from different sources such as Paddy Breeding Station (PBS), International Rice Research Institute, Harvest Plus lines from Indian Institute of Rice Research, CHIR and ARB lines from West Bengal and Karnataka (Annexure I).

Experimentation

The study was undertaken at Department of Rice, Tamil Nadu Agricultural University, Coimbatore during *Kharif* 2015. Crop was raised under irrigated condition in an area of 26 cents with the recommended fertilizer dosage of 150:60:60 Kg/ha of NPK. Once it attained physiological maturity, plants were harvested; seeds cleaned and dried upto the moisture level of 13%. The dried seeds were dehusked using hand palm dehusker, ground evenly and sieved upto the talcum powder size with pestle and mortar. The powdered samples were used for sample preparation.

Determination of Folate content in grain samples

Folate content in the rice grain sample was quantified by the method of Poo-Prieto *et al.*, (2006).

Extraction of folate

Thawed samples were suspended (1 g/10 ml final volume) in 0.026 mol Tris-HCl extraction buffer (pH 7.4) containing 1% (w: v) sodium ascorbate. The cap covered samples were autoclaved for 15 min at 120^{o}C (1.034 bar) and cooled in ice bath. The homogenates were subjected to a modified tri-enzyme treatment method followed by Martin *et al.*, (1990). Homogenates incubated for 4 h at 37 ^{o}C were added with 1.25 ml of α-amylase solution. Around 0.2 ml of folate conjugase was added to deconjugate the polyglutamates to monoglutamates. Subsequently, the samples were treated with 1 ml protease to release the folate from folate binding proteins and simultaneously denature the enzymes that may catalyze degradation or inter conversion of folate (Pfeiffer *et al.*, 1997). Enzyme treated samples were incubated in a boiling water bath for 5 min and cooled in ice bath. Chilled samples were then centrifuged at 36,000 rpm for 20 min at 4^{o}C. The supernatants were filtered through a membrane filter of 0.02 µm pore size and it was stored at - 80^{0}C overnight for further analysis.

Preparation of standards

For the preparation of folate standard, folic acid was obtained from Sigma Aldrich. Standard stock solutions were prepared by dissolving 20 µmol folic acid in 0.01 mol NaOH separately. Concentration of folate was determined at 280 nm UV absorption with the pH 7.0 buffer solutions and a molar extension coefficient of 27600L. Following spectral determinations, the standard stock solutions were stored in small aliquots in 1% sodium ascorbate at -20^{o}C. These standards were further purified by affinity column chromatography before use.

Preparation of enzymes

Folate conjugase was dissolved in Phosphate buffer at the concentration of 1:500. α-amylase and protease obtained from Sigma Aldrich were dissolved in water at a final concentration of 20 mg protein/ml and 2 mg protein/ml respectively.

HPLC measurement of folate

A flow rate of 1 ml/min was used for the column separation. The mobile phase program consisted of 3 min with 100% solution A (28 mmol dibasic potassium phosphate and 60 mmol phosphoric acid in water) followed by a linear gradient of 10 min with 70% A: 30% solution B (28 mmol dibasic potassium phosphate and 60 mmol phosphoric acid in 200 ml acetonitrile and 800 ml water). A second linear gradient using 70% A: 30% B for 10 minutes followed by 45% A: 55% B for seven minutes was then run for the next 17 min. This was followed by

a third linear gradient of 43% A: 57% B for the next 15 min. At 45 min, the column was equilibrated for 5 min to the initial conditions and another sample analysis could be initiated immediately.

The absorbance of folic acid was monitored with a UV detector set at 280 nm. Peak identification was detected, based on a combination of the retention time and the spectral characteristics.

Quantification

Quantification was based on the external standard method in which UV peak (folic acid) was plotted against concentration. The standard solutions were purified through FBP–Affigel 10 affinity columns and the calibration curves were prepared from those using similar standard levels as expected in the samples. Folic acid concentration was calculated using the following formula **(**Indrasari *et al.*, 2013).

Folic acid (µ g 100 g $^{-1}$) = SC from standard curve x DF x 100

Where,

SC- Sample Concentration
DF- Dilution Factor

Results and Discussion

Rice is the major staple food for Asian countries contributing high amount of carbohydrate for more than 50 % of the human population. The disadvantage of this nature of food habit is that people become deficient in one or more vitamin complexes (World Food Programme, 2015). Nearly 3 billion people were affected by malnutrition which proceeds towards infant mortality (Gearing, 2015). In this present situation, attention on grain quality and nutritional value has become an important goal for plant breeders. Rice grain has low level of folate when compared to other staple crops, legumes and tubers (Adeyeye *et al.*, 2000).

Nearly 50 nutrients are indispensable for executing metabolic activities in human being (Welch and Graham, 2004). Among those nutrients, folate governs most of the basic metabolic activities required for human health. To enhance the folate level, the primary objective of breeders is to study the available folate accumulation in rice grains. However, as limited information is available on folate content in brown rice, our present study was aimed to explore the diversity of folate pool in 150 germplasm accessions of rice.

Total folate content of brown rice

Folate are abundant in green leafy vegetables (folium is Latin for leaf), such as spinach and legumes (e.g. beans). Most staple crops, such as rice and other cereals, contain very low amounts of this vitamin. Rice crop has a huge collection of germplasm lines which have served as donors for different traits. So, screening germplasm for folate content is the primary step for choosing parents to inculcate in breeding programmes.

Quantification of Vitamin B9 is difficult due to instability and the complexity of its form. Folate can be quantified by various methods namely, microbiological assay, HPLC coupled to fluorescence or UV detectors (Gregory, 1989; Doherty and Beecher, 2003; Zhang *et al.*, 2003), electrochemical detectors (Bagley and Selhub, 2000).

The microbiological assay using *Lactobacillus rhamnosus* as the test organism (Keagy, 1985) was used in the past which relies on turbedimetric bacterial growth (Hawkes and Villota, 1989). The main

drawbacks of this method were the influence of non-folate substances in the growth of test organism and also improper maintenance of cultures. Because of the tedious process also accompanied with drawbacks, chromatographic methods were developed which consists of extraction, detection and quantification.

HPLC is considered as a highly accurate method to estimate even the negligible amounts of folate (Vahteristo *et al.,* 1996). In cereal-grain products, the gradient HPLC method adopted by Pfeiffer *et al.,* (1997) allowed a good separation of folates within 33 min run time.

In our study, folates were separated at 45 minutes run time from powdered brown rice. Detection of folate molecules using HPLC C18 columns have been commonly established with UV absorbance (Doherty and Beecher, 2003).

Various findings have revealed that the tri-enzyme technique is an ideal extraction method for complete extraction of folates trapped in complex carbohydrate or protein structures in carbohydrate and protein rich foods (Desouza and Eteinmiller, 1990; Tamura *et al.,* 1997; Rader *et al.,* 1998). Folate activity was represented by peak activity observed in UV analytical column (Bagle and Selhub, 1997).

In our study, the representative chromatographic result of folic acid standard observed at 280nm UV absorption is shown in Figure 1. Based on the standard graph, 1.2 millivolt/ second was arrived as the specific retention time for estimating folate pool in the germplasm accessions.

On an average, three samples per accession were estimated for folate and the mean values were arrived (Table 1). The overall folate concentrations of the tested samples ranged from 9.72 µg/ 100g in accession RG106 (Katta samba) to 29.28 µg /100g in accession RG2 (CK 275) and are depicted in Figure 2a and 2b. This represents a threefold difference between the lowest and the highest folate concentration.

One hundred and twenty three genotypes had the folate concentration between 12.01 and 19.00 µg / 100g of sample which is considered as moderate level of folate among the tested gene pool.

About 6.66% (10 accessions) of the accessions had low folate content of less than 12 µg/100g of sample and 11.34% (17 accessions) of accessions recorded high folate content of more than 19.01 µg/ 100g of sample.

The experimental CV was 2.92% and forty one germplasm lines recorded significantly higher folate content than the grand mean of 16.05 µg /100g. Based on the frequency distribution graph, we conclude that most of the accessions fall in the category of moderate folate content (Fig. 3).

On the basis of manual grouping of accessions (Table 2), germplasm with above 19.01µg/100g of folate content were grouped as high folate pool which comprised of 17 genotypes namely RG1 (Mapillai samba), RG2 (CK275), RG3 (Senkar), RG4 (Murugankar), RG 162 (IR 64), RG5 (CHIR 6), RG150 (IG 14), RG12 (Vellai chithiraikar), RG52 (ARB 58), RG14 (Jyothi), RG74 (ARB 65), RG20 (Kalvalai), RG32 (Thogai samba), RG17 (Chivapu chithiraikar), RG42 (Earapalli samba), RG39 (Kaatu ponni) and RG22 (IR 36). Germplasm with folate range of 12.01-19.00 µg/100g and less than 12 µg/100g were grouped as moderate and low folate content respectively among the accessions studied.

Table.1 Mean folate content of rice Association Mapping Panel

S. No.	Acc.No	Mean	S. No.	Acc.No	Mean	S. No.	Acc.No	Mean	S. No.	Acc.No	Mean
1.	RG1	27.942**	24.	RG36	11.338	47.	RG65	17.129**	70.	RG99	16.929*
2.	RG2	29.284**	25.	RG37	10.213	48.	RG66	15.139	71.	RG100	15.812
3.	RG3	27.794**	26.	RG39	22.453**	49.	RG67	14.139	72.	RG101	15.823
4.	RG4	28.164**	27.	RG41	15.413	50.	RG68	11.600	73.	RG102	14.395
5.	RG5	19.679**	28.	RG42	22.969**	51.	RG69	10.802	74.	RG103	13.409
6.	RG6	18.603**	29.	RG43	13.672	52.	RG70	13.728	75.	RG104	13.671
7.	RG7	16.416	30.	RG44	15.469	53.	RG71	12.516	76.	RG105	15.438
8.	RG8	17.087**	31.	RG45	13.083	54.	RG72	15.229	77.	RG106	9.721
9.	RG9	17.743**	32.	RG46	16.393	55.	RG74	20.337**	78.	RG107	15.789
10.	RG12	19.055**	33.	RG48	14.819	56.	RG76	10.307	79.	RG108	16.933*
11.	RG14	20.367**	34.	RG50	16.177	57.	RG77	15.19	80.	RG109	15.252
12.	RG15	18.316**	35.	RG51	15.821	58.	RG80	16.124	81.	RG110	17.8**
13.	RG17	23.229**	36.	RG52	19.174**	59.	RG81	15.55	82.	RG112	15.201
14.	RG18	15.693	37.	RG53	13.927	60.	RG82	17.183**	83.	RG113	13.677
15.	RG20	20.011**	38.	RG54	16.562	61.	RG83	18.251**	84.	RG114	13.313
16.	RG22	23.944**	39.	RG55	13.457	62.	RG85	15.81	85.	RG115	12.956
17.	RG25	13.781	40.	RG56	17.296**	63.	RG86	18.106**	86.	RG116	14.355
18.	RG26	15.101	41.	RG57	16.958*	64.	RG89	12.952	87.	RG117	13.502
19.	RG31	15.404	42.	RG58	13.633	65.	RG91	13.662	88.	RG118	13.574
20.	RG32	21.008**	43.	RG59	11.231	66.	RG92	13.995	89.	RG120	13.689
21.	RG33	15.388	44.	RG60	18.586**	67.	RG95	12.695	90.	RG121	17.536**
22.	RG34	17.43**	45.	RG62	17.24**	68.	RG96	13.26	91.	RG122	12.616
23.	RG35	13.216	46.	RG63	15.735	69.	RG98	16.979*	92.	RG123	14.081

* Significant at 0.05% level; ** Significant at 0.01% level

Table.1 Cont.…

S. No.	Acc.No	Mean	S. No.	Acc.No	Mean	S. No.	Acc.No	Mean
93	RG124	9.959	113	RG149	16.004	133	RG173	13.491
94	RG126	15.544	114	RG150	19.609**	134	RG174	13.535
95	RG127	15.999	115	RG151	14.67	135	RG175	15.499
96	RG128	17.062**	116	RG152	16.407	136	RG176	15.522
97	RG129	16.356	117	RG154	16.266	137	RG178	14.053
98	RG130	15.627	118	RG156	15.715	138	RG180	13.47
99	RG131	16.014	119	RG157	18.441**	139	RG181	14.748
100	RG132	15.406	120	RG158	14.509	140	RG182	13.411
101	RG133	11.901	121	RG159	13.631	141	RG183	16.161
102	RG134	13.855	122	RG160	16.063	142	RG184	17.007
103	RG135	15.034	123	RG161	16.023	143	RG185	14.896
104	RG136	16.888*	124	RG162	27.521**	144	RG186	14.86
105	RG137	18.168**	125	RG163	14.541	145	RG187	17.036
106	RG141	14.421	126	RG164	16.622	146	RG188	16.086
107	RG142	15.439	127	RG165	17.263**	147	RG189	15.585
108	RG143	11.857	128	RG166	18.137**	148	RG190	15.915
109	RG145	15.871	129	RG168	13.915	149	RG191	15.428
110	RG146	15.282	130	RG169	14.622	150	RG192	15.984
111	RG147	15.422	131	RG170	14.294			
112	RG148	13.801	132	RG172	13.462			
Grand mean								16.05
Range								9.7 -29.28
SE (d)								0.38
CD (0.05%)								0.75
CD (0.01%)								0.99
CV%								2.92

* Significant at 0.05% level; ** Significant at 0.01% level

Table.2 Manual grouping of rice germplasm based on folate content

Folate concentration µg/100g	Number of individuals	Accession number	Percentage of individuals	*Grouping
>19.01	17	RG1,RG2,RG3,RG4,RG5,RG12,RG14,RG17,RG20,RG22,RG32, RG39,RG42,RG52,RG74,RG150,RG162	11.34	High
12.01 -19.00	123	RG6,RG7,RG8,RG9,RG15,RG18,RG26,RG31,RG33,RG34,RG41,RG44, RG46,RG48,RG50,RG51,RG54,RG56,RG57,RG60,RG62,RG63,RG65,RG66,RG72, RG77,RG80,RG81,RG82,RG83,RG85,RG86,RG98,RG99,RG100, RG101,RG105,RG107,RG108,RG109,RG110,RG112,RG121,RG126,RG127,RG128, RG129,RG130,RG131,RG132,RG135,RG136,RG137,RG142,RG145,RG146,RG147, RG149,RG151,RG152,RG154,RG156,RG157,RG160,RG161,RG164,RG165,RG166, RG169,RG175,RG176,RG181,RG183,RG184,RG185,RG186,RG187,RG188, RG189,RG190,RG191,RG192	82.00	Moderate
<12.00	10	RG36,RG37,RG59,RG69,RG68,,RG76,RG106,RG124RG133,RG143	6.66	Low
*Grouping >19.01µg/100g –High ; 12.01 to19.00 µg/100g- Moderate ; <12.00 µg/100g - Low				

Table.3 Grouping and clustering of germplasm using UPGMA method

Main cluster	Sub cluster	Cluster Range (µg/100g)	Number of individuals	Accession number	*Grouping
I	-	29.284 to 27.521	5	RG1,RG2,RG3,RG4,RG162	High
II	1	19.055 to 21.008	8	RG5, RG12, RG14, RG20,RG32, RG52, RG74,RG150	High
	2	23.944 to 22.453	4	RG17, RG22, RG39, RG42	High
III	1	12.516 to 18.603	123	RG6,RG7,RG8,RG9,RG15,RG18,RG26,RG31,RG33,RG34,RG41, RG44,RG46,RG48,RG50,RG51,RG54,RG56,RG57,RG60,RG62, RG63,RG65,RG66,RG72,RG77,RG80,RG81,RG82,RG83,RG85, RG86,RG98,RG99,RG100,RG101,RG105,RG107,RG108,RG109, RG110,RG112,RG121,RG126,RG127,RG128,RG129,RG130, RG131,RG132,RG135,RG136,RG137,RG142,RG145,RG146, RG147,RG149,RG151,RG152,RG154,RG156,RG157,RG160, RG161,RG164,RG165,RG166,RG169,RG175,RG176,RG181, RG183,RG184,RG185,RG186,RG187,RG188,RG189,RG190, RG191,RG192	Moderate
	2	9.721 to 11.901	10	RG36, RG37,RG59,RG69,RG68,RG76,RG106,RG124,RG133,RG143,	Low
*Grouping >19.01µg/100g –High ; 12.01 to19.00 µg/100g- Moderate ; <12.00 µg/100g - Low					

Figure.1 Chromatograph of standard folic acid at UV detection at 280 nm

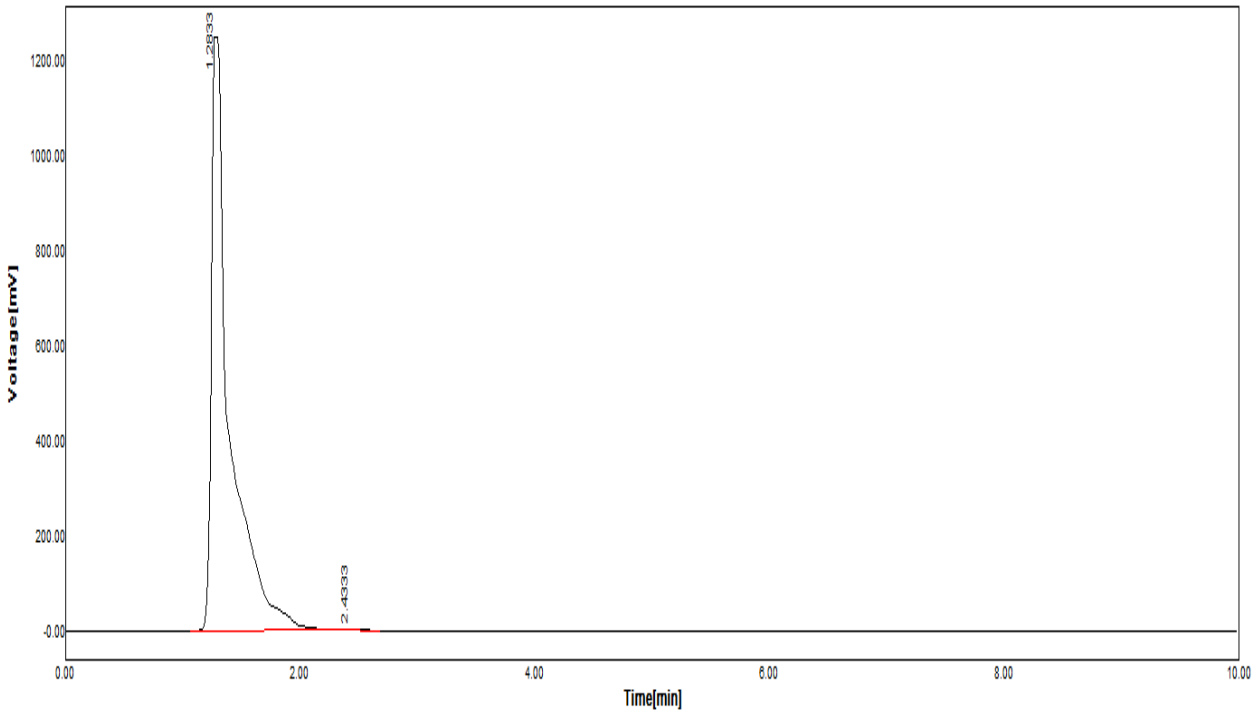


Figure.2a Chromatograph of RG 2 (CK 275) at UV detection at 280 nm

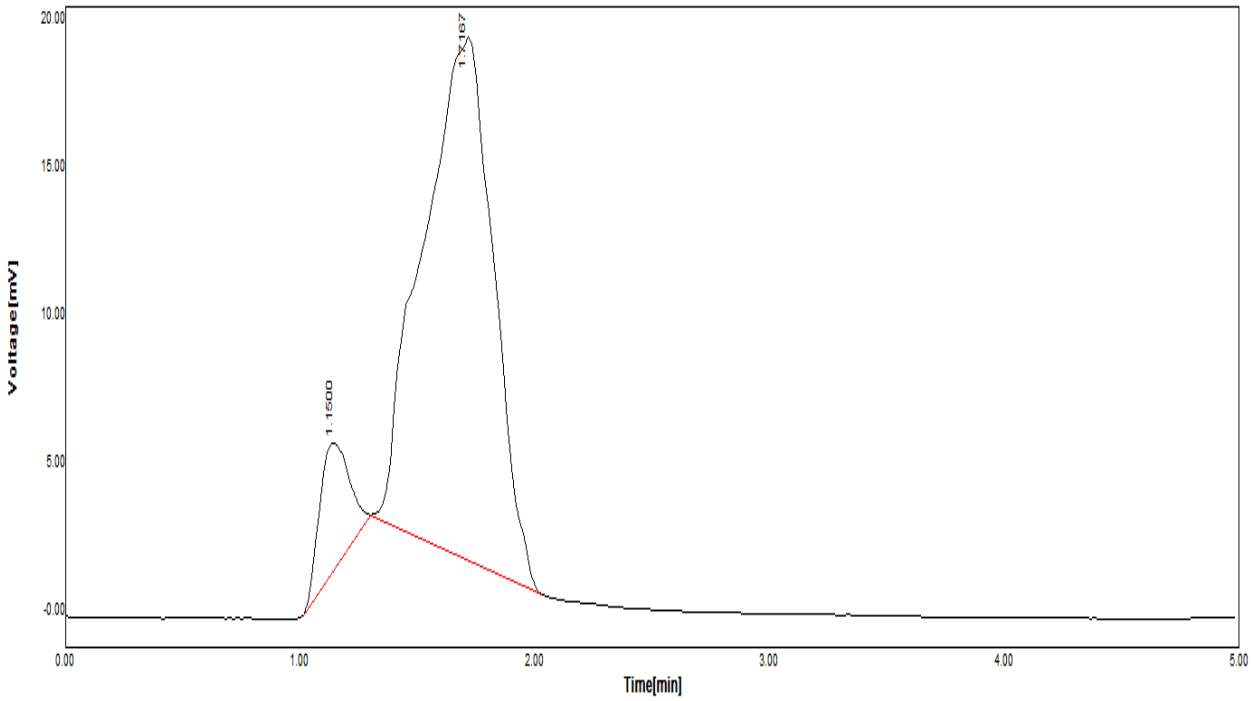


Figure.2b Chromatograph of RG106 by UV detection at 280 nm (low folate)

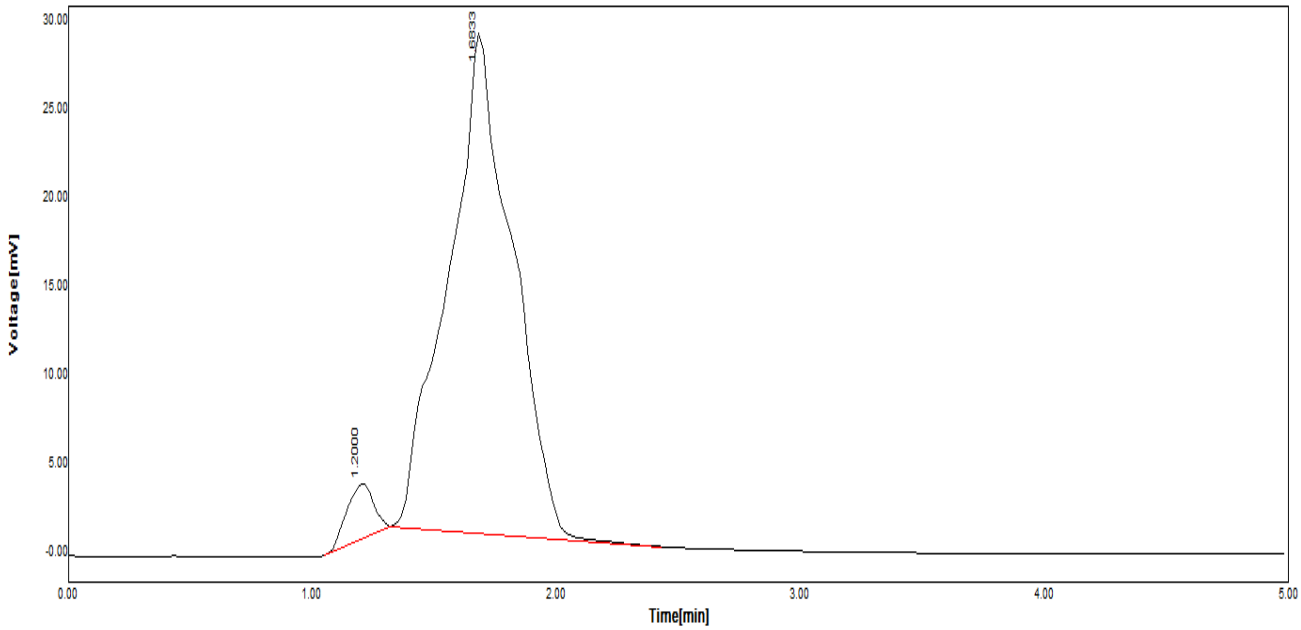


Figure.3 Frequency distribution of folate content among 150 germplasm accessions

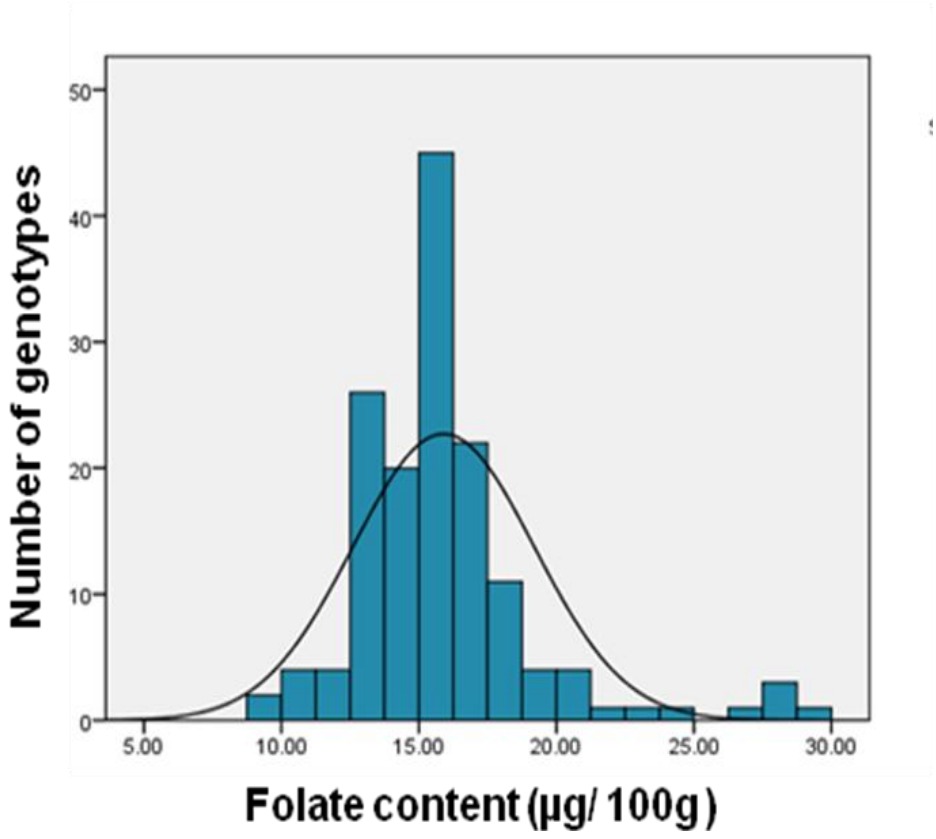
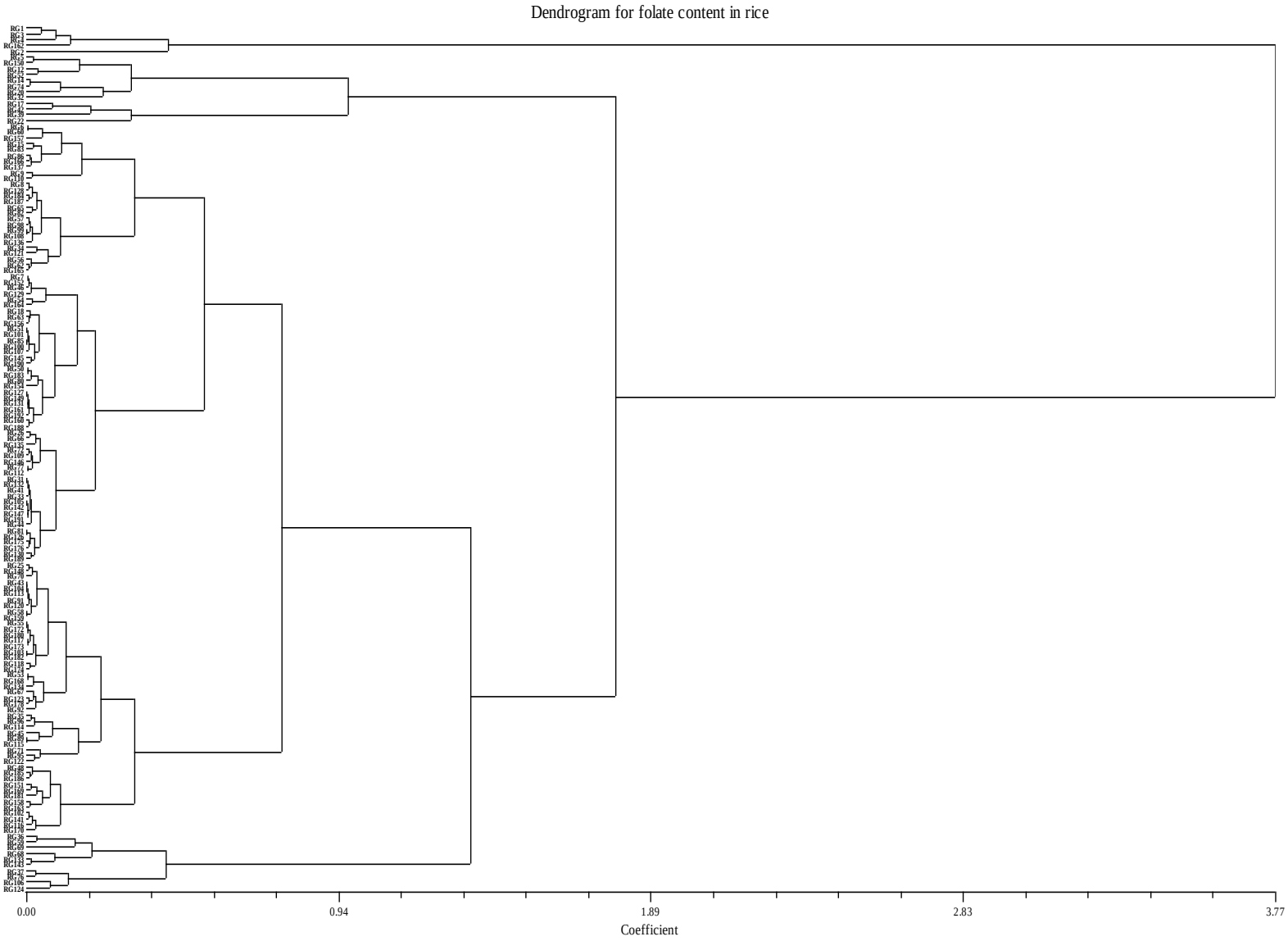


Figure.4 UPGMA dendrogram showing three clusters (I, II and III) of 150 rice genotypes at the similarity coefficient of 1.70



Brouwer *et al.*, (2008) reported the total folate content of freshly harvested seeds of *japonica* Nipponbare as 44.4±2.9g/100 g by Liquid chromatography mass spectrometric method. Folate content was estimated by microbiological assay and chromatographic methods in 51 rice cultivars by Riza *et al.*, (2010). There were differences in folate content between the two methods. The total folate content in their study ranged from 6.7 to 86.6 µg/100g (Balam, from Bangladesh). Most of the elite *indica*, irrigated lowland and basmati cultivars had <50 µg/100g total folate as per microbiological assay. In HPLC-UV method, Moroberekan had the highest content of 58.9 µg/100g (value was 63.6 µg/100g in microbiological assay) followed by IR 72 (42.2 µg/100g) and IR 64 (27.2 µg/100g). The folate content of IR 64 is almost similar to our present report (RG162 – IR 64- 27.52 µg/100g).

In a study of 78 rice cultivars consisting of 39 *indica* and 39 *japonica* accessions, Dong *et al.*, (2011) reported 8.4 fold difference between the lowest (Longhuamaohulu, 13.3 µg/100 g) and the highest (Chaoyangzao 18, 111.4µg/100 g) folate concentrations in brown rice which is unlike our study where there is only a three-fold difference. Their method of estimation was through microbiological assay but still higher values were obtained than the normal range of 8.0 to 44.4 µg/100 g reported by independent researchers using HPLC or microbiological assays earlier (Pfeiffer *et al.*, 1997; Konings *et al.*, 2001; Yon and Hyun, 2003; Rychlik, 2004; Brouwer *et al.*, 2008; Vishnumohan *et al.*, 2009). The differences were attributed to have been caused by the growing environment, folate extraction method or assay method.

Microbiological assays have poor precision and fail to differentiate between several folates. In general, most HPLC-measured folate contents were lower than values found by microbiological assay. The reason is that non folate compounds influence the bacterial growth response, resulting in higher folate contents with the microbiological assay (Konings *et al.*, 2001). The general folate level of brown rice is 20 µg/100 g as reported by Saskia de Pee (2014).

Diversity of rice germplasm accessions

The dendrogram of folate content (Fig. 4) derived through cluster analysis using NTSys 2.0 software divided the germplasm accessions into three major clusters at the similarity coefficient of 1.70 (Table 3).

Cluster I comprised of five genotypes namely RG1 (Mapillaisamba), RG2 (CK275), RG3 (Senkar), RG4 (Murugankar) and RG162 (IR 64) with the folate range of 27.521 - 29.28 µg/100g of ground unpolished rice and very high among the accessions studied.

Cluster II comprised of twelve genotypes falling in the category of high folate pool with two sub clusters. Sub cluster I had eight accessions with folate content ranging from 19.05 - 21.08 µg/100g whereas, sub cluster II possessed four genotypes *viz.*, RG17 (Sivapuchithiraikar), RG42 (Earapallisamba), RG39 (Kaatuponni) and RG22 (IR36) with the folate range of 22.453-23.969 µg/100g.

Cluster III possessed two sub clusters comprising of 133 genotypes in total. Sub cluster I had lines with moderate level of folate ranging from 12.51 -18.60 µg/100g. Sub cluster II of main cluster III accommodated ten genotypes namely RG36 (Kattikar), RG59 (RPHP68), RG69 (RPHP48), RG68 (IG63), RG133 (IG42), RG143 (IG46), RG37 (Shenmolagai), RG76 (Mattakuruvai), RG106 (Kattasamba) and RG124 (IG29) with low level of folate ranging from 9.72-11.90 µg/100g.

Among the different subspecies, *indica* contained 32% higher folate concentrations than *japonica* in brown rice. Hence, Dong *et al.,* (2011) suggested a tendency for geographical origin to be related to high folate and implies a genetic component of variation. In our study, out of five genotypes in cluster I with high folate content, three *viz.,* Mapillai samba (RG1), Senkar (RG3) and Murugankar (RG4) are landraces of Tamil Nadu. The genotype RG2 (CK 275) is an improved breeding line from a cross between a well-adapted variety CO (R) 50 with a landrace named Kavuni which is rich in dietary fiber, phenolic acids, flavanoids, iron, calcium and other minerals (Valarmathi *et al.,* 2014)

HPLC estimation of folate allows detection of even negligible quantities of folate content in the given sample and is of high resolution. The present study described the quantitative estimation of folate in brown rice in 150 genotypes of an association mapping panel using HPLC method which revealed a three - fold variation. Diversity analysis indicated that accessions from high and low folate pool can be utilized in recombination breeding to enhance the level of folate and also to map the genomic regions for enhanced folate content. Such enhanced levels of folate was encountered by Dong *et al.,* (2014) in a set of 264 F_{12} recombinant inbred lines (RILs) derived from a cross between varieties Lemont (*japonica,* of low folate content) and Teqing (*indica*, with high content of 107.9 µg/100 g) analyzed for folate content in two years. Their study revealed that folate content ranged from 10.0 to 62.5 µg/100g in 2008 and 25.2 to 169.4 µg/100g in 2010. Since rice is the staple food, nutritional enhancement of rice grain is the primary objective of researchers to provide nutritional security. Genomic strategies with conventional breeding will give additional value to enhance nutritional quality in rice when it moves hand in hand.

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Annexure.I Details of 150 rice Association mapping panel used in the study

G.No	Genotypes	Parentage	Origin
RG1	Mapillai samba	Landrace	Tamil Nadu, India
RG2	CK 275	CO(R) 50 x Kavuni	Tamil Nadu, India
RG3	Senkar	Landrace	Tamil Nadu, India
RG4	Murugankar	Landrace	Tamil Nadu, India
RG5	CHIR 6	Improved chinsurah	West Bengal
RG6	CHIR 5	Improved chinsurah	West Bengal
RG7	Kudai vazhai	Landrace	Tamil Nadu, India
RG8	CHIR 8	Improved chinsurah	West Bengal
RG9	Kuruvai kalanjiyam	Landrace	Tamil Nadu, India
RG12	Vellai chithiraikar	Landrace	Tamil Nadu, India
RG14	Jothi	Variety	Kerala,India
RG15	Palkachaka	Landrace	Tamil Nadu, India
RG17	Sivapuchithiraikar	Landrace	Tamil Nadu, India
RG18	CHIR 11	Improved chinsurah	West Bengal
RG20	Kalvalai	Landrace	Tamil Nadu, India
RG22	IR 36	IR1561-228-L2/IR1737/ /CR94-13	IRRI, Philippines
RG25	Sorna kuruvai	Landrace	Tamil Nadu, India
RG26	Rascadam	Landrace	India
RG31	Chinthamani	Landrace	Tamil Nadu, India
RG32	Thogai samba	Landrace	Tamil Nadu, India
RG33	Malayalathan samba	Landrace	Tamil Nadu, India
RG34	RPHP 125	NDR 2026(RICHA)	Uttarpradesh, India
RG35	CK 143	CO(R) 50 x Kavuni	Tamil Nadu, India
RG36	Kattikar	Landrace	Tamil Nadu, India
RG37	Shenmolagai	Landrace	Tamil Nadu, India
RG39	Kaatu ponni	Landrace	Tamil Nadu, India
RG41	Godavari samba	Landrace	India
RG42	Earapalli samba	Landrace	India
RG43	RPHP 129	Kamad	Jammu and Kashmir
RG44	Mangam samba	Landrace	Tamil Nadu, India
RG45	RPHP 105	Moirang phou	Manipur, India
RG46	IG 4 (EC 729639- 121695)	TD2: : IRGC 9148-1	IRRI, Philippines
RG48	Kalarkar	Landrace	Tamil Nadu, India
RG50	Sornavari	Landrace	Tamil Nadu, India
RG51	RPHP 134	Njavara	Kerala,India
RG52	ARB 58	Variety	Karnataka, India
RG53	IR 68144-2B-2-2-3-1-127	IR72 x Zawa bonday	IRRI, Philippines

RG54	PTB 19	Variety	Kerala, India
RG55	IG 67(EC 729050- 120988)	IR77384-12-35-3-12-1-B::IRGC117299-1	IRRI, Philippines
RG56	RPHP 59	Taroari Basmati/Karnal local	Haryana, India
RG57	RPHP 103	Pant Sugandh Dhan 17	Uttarkhand, India
RG58	Kodaikulathan	Landrace	Tamil Nadu, India
RG59	RPHP 68	Subhadra	Orissa, India
RG60	Rama kuruvaikar	Landrace	Tamil Nadu, India
RG62	Purpleputtu	Landrace	Tamil Nadu, India
RG63	IG 71(EC 728651- 117588)	TEPI BORO: :IRGC 27519-1	IRRI, Philippines
RG65	IG 56 (EC 728700- 117658	Bico branco	IRRI, Philippines
RG66	Seevansamba	Landrace	Tamil Nadu, India
RG67	RPHP 106	Akut phou	Manipur, India
RG68	IG 63(EC 728711- 117674)	Caawa/Fortuna	IRRI, Philippines
RG69	RPHP 48	Bindli	Uttarkhand, India
RG70	Karthi samba	Landrace	Tamil Nadu, India
RG71	IG 27(IC0590934- 121255)	ARC11345: :IRGC21336 -1	IRRI, Philippines
RG72	Aarkadu kichili	Landrace	Tamil Nadu, India
RG74	ARB 65	Variety	Karnataka, India
RG76	Matta kuruvai	Landrace	Tamil Nadu, India
RG77	Karuthakar	Landrace	Tamil Nadu, India
RG80	IG 66(EC 729047- 120985)	IR71137-243-2-2-3-3: : IRGC 99696 -1	IRRI, Philippines
RG81	CB 07701-252	Improved line	Tamil Nadu, India
RG82	Thooyamalli	Landrace	Tamil Nadu, India
RG83	RPHP 93	Type-3 Dehraduni basmati	Uttarkhand, India
RG85	RPHP 104	Kasturi (IET8580)	Uttarkhand, India
RG86	RPHP 102	Kanchana	Kerala,India
RG89	IR 83294-66-2-2-3-2	Daesanbyeo x IR65564-44-5-1	IRRI, Philippines
RG91	IG 23(EC 729391- 121419)	Mahapannithi ::IRGC51021-1	IRRI, Philippines
RG92	IG 49(EC 729102- 121052)	Menakely::IRGC69963-1	IRRI, Philippines
RG95	Jeeragasamba	Landrace	Tamil Nadu, India
RG96	RP BIO 226	BPT5204*4/SS1113	AndhraPradesh, India
RG98	IG 5(EC 729642- 121698)	IR65907-116-1-B::C1	IRRI, Philippines
RG99	IG 31(EC 728844- 117829)	Oryzica llanos 5	Colombia
RG100	IG 7(EC 729598- 121648)	Vary manity:: IRGC69910-1	IRRI, Philippines
RG101	RPHP 52	Sebati	Odisha, India
RG102	Varakkal	Landrace	Tamil Nadu, India
RG103	Mattaikar	Landrace	Tamil Nadu, India
RG104	IG 53(EC 728752- 117719)	Carolina rinolda barsani	Uruguay
RG105	IG 6 (EC 729592- 121642)	SOM CAU 70 A::IRGC8227-1	IRRI, Philippines

RG106	Katta samba	Landrace	Tamil Nadu, India
RG107	RH2-SM-1-2-1	Swarna x Moroberekan	Tamil Nadu, India
RG108	Red sirumani	Landrace	Tamil Nadu, India
RG109	Vadivel	Landrace	Tamil Nadu, India
RG110	Norungan	Landrace	Tamil Nadu, India
RG112	IG 35(EC 728858- 117843)	Pate blanc MN1	Cote D'ivoire
RG113	IG 45(EC 728768- 117736)	Fortuna	Puerto Rico
RG114	RPHP 159	Radhuni pagal	Bangladesh
RG115	IG 43(EC 728788- 117759)	-	IRRI, Philippines
RG116	RPHP 27	Azucena	IRRI, Philippines
RG117	IG 65(EC 729024- 120958)	GODAHEENATI:: IRGC31393-1	IRRI, Philippines
RG118	Ponmani samba	Landrace	Tamil Nadu, India
RG120	Thattan samba	Landrace	Tamil Nadu, India
RG121	IG 74(EC 728622- 117517)	Kinandang Patong::IRGC23364-1	IRRI, Philippines
RG122	Kaliyan samba	Landrace	Tamil Nadu, India
RG123	IG 2(EC 729808-121874)	BLUEBONNET 50::IRGC1181	IRRI, Philippines
RG124	IG 29(EC 728925- 117920)	TO x782-20-1	IRRI, Philippines
RG126	Kallimadayan	Landrace	Tamil Nadu, India
RG127	IG 10(EC 729686- 121743)	Hasan seralirGC79564 -C1	IRRI, Philippines
RG128	IG 75(EC 728587- 117420)	Aedal:: IRGC55441-1	IRRI, Philippines
RG129	IG 38(EC 728742- 117707)	Delrex	United states
RG130	IG 39(EC 728779- 117750)	Honduras	Honduras
RG131	RPHP 90	24(K)	Andhra Pradesh, India
RG132	IG 33(EC 728938- 117935)	WC3397	Jamaica
RG133	IG 42(EC 728798- 117774)	Kalubala vee	Srilanka
RG134	IG 9(EC 729682- 121739)	Gemjya jyanam::IRGC32411-C1	IRRI,Philippines
RG135	RPHP 161	Champa khushi	WestBengal,India
RG136	IG 8(EC 729601- 121651)	Xi you zhan :: IRGC78574-1	IRRI,Philippines
RG137	IG 37(EC 728715- 117678)	Cenit	Argentina
RG141	IG 44(EC 728762- 117729)	Edith	United states
RG142	Sasyasree	Variety (TKM 6 x IR 8)	West Bengal, India
RG143	IG 46(IC 471826- 117647)	Baber	India
RG145	IG 60(EC 728730- 117695)	Creole	Belize
RG146	IR 75862-206	IR75083 x IR6565600-81-5-3-2	IRRI,Philippines
RG147	IG 58(EC 728725- 117689)	CI 11011	United states
RG148	Chinna aduku nel	Landrace	Tamil Nadu, India
RG149	RH2-SM-2-23	Swarna x Moroberekan	Tamil Nadu, India
RG150	IG 14(IC 517381- 121422)	Malachan::IRGC54748-1	IRRI, Philippines
RG151	IG 32(EC 728838- 117823)	NOVA	United states

RG152	RPHP 47	Pathara	Orissa, India
RG154	IG 48(EC 729203- 121195)	Dinolores::IRGC67431-1	IRRI, Philippines
RG156	IG 12(EC 729626- 121681)	Shestak::IRGC32351-1	IRRI, Philippines
RG157	Karungan	Landrace	Tamil Nadu, India
RG158	IG 13(EC 729640- 121696)	Curinca::C1	IRRI, Philippines
RG159	Sembala	Landrace	Tamil Nadu, India
RG160	IG 72(EC 728650- 117587)	TD 25::IRGC 32351-1	IRRI, Philippines
RG161	Panamarasamba	Landrace	Tamil Nadu, India
RG162	IR 64	IR 5857-33-2-1 x IR 2061-465-1-5-5	IRRI, Philippines
RG163	Mikuruvai	Landrace	Tamil Nadu, India
RG164	Thillainayagam	Landrace	Tamil Nadu, India
RG165	ARB 64	Variety	Karnataka, India
RG166	RPHP 140	Vytilla anakondan	Kerala,India
RG168	Haladichudi	Landrace	Odisha, India
RG169	IG 24(EC 728751- 117718)	DNJ 140	Bangladesh
RG170	RPHP 42	Shalimar rice-1	Jammu and Kashmir
RG172	IG 25(EC 729728- 121785)	Lohambitro 224::Gervex 5144-C1	IRRI, Philippines
RG173	IG 73(EC 728627- 117527)	Makalioka 34::IRGC 6087-1	IRRI, Philippines
RG174	IG 51(EC 728772- 117742)	Gogo lempuk	Indonesia
RG175	Vellai kudaivazhai	Landrace	Tamil Nadu, India
RG176	Kodai	Landrace	Tamil Nadu, India
RG178	IG 17(EC 728900- 117889)	Sigadis	Indonesia
RG180	IG 59(EC 728729- 117694)	Coppocina	Bulgaria
RG181	IG 52(EC 728756- 117723)	Dourado agulha	Brazil
RG182	ARB 59	Variety	Karnataka, India
RG183	RPHP 163	Seeta sail	WestBengal, India
RG184	IG 18(EC 728892- 117880)	Seratoes hari	Indonesia
RG185	RPHP 36	Variety, TKM 9	Tamil Nadu, India
RG186	IG 28(EC 728920- 117914)	Tia bura	Indonesia
RG187	Vadakathi samba	Landrace	Tamil Nadu, India
RG188	RPHP 80	24(K)	Andhra Pradesh, India
RG189	IG 41(EC 728800- 117776)	Kaniranga	Indonesia
RG190	IG 26(IC0590943- 121899)	Basmati 370 ::IRGC 3750-1	IRRI, Philippines
RG191	IG 15(EC 728910- 117901)	Sze guen zim	China
RG192	Nootri pathu	Landrace	Tamil Nadu, India