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Waxy gene polymorphism and its association with grain quality traits in selected landraces of rice

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ABSTRACT

The quality breeding of rice is mainly based on the amylose content, which is in turn determined by waxy gene locus. The genetic polymorphism of the Wx gene in 82 rice landraces and varieties were studied using a simple sequence repeat (SSR) marker RM 190. The genotypes were studied for amylose content, grain length, grain breadth, LB ratio, grain colour, alkali spreading value and gelatinisation temperature. Based on amylose content, the genotypes were classified into different categories as low amylose (10-20% amylose), intermediate amylose (20-24% amylose) and high amylose (>25% amylose). The amylose content ranged from 14.22% (Ganthasala) to 33.6% in (Vadivel). The employment of SSR markers in genetic diversity analysis also helped in grouping the genotypes on amylose content. The SSR primer, RM190 showed 48.95% correlation with phenotypical variation of amylose in the selected landraces.

Key words: *rice, quality, amylose content, SSR markers, waxy gene*

Rice is the important food crop for majority of world population. Rice grain quality has received increasing attention in recent years especially in Asia. With the advance of standard of living, people have different demands for rice grain quality in terms of its various applications in food and industrial sector. Regional variation in preference for rice quality is also observed. Cooking and consumption quality is one of the most important components of grain quality. Cultivars with different grain qualities are also required for medicinal, ceremonial, or special production purposes (Tian *et al.,* 2009). Grain quality has now become one of the primary considerations of rice customers and breeders.

During the development of rice grain the endosperm accumulates starch and storage proteins and occupies the major part of the grain (about 90%). Based on these storage proteins rice has been classified into glutinous and non glutinous. Rice starch is made up of glucose polymers like highly branched amylose and relatively unbranched amylopectin (Juliano, 1985). Low amylose content is usually associated with tender, cohesive and glossy cooked rice and high amylose content results in hard texture and low viscosity with good swelling capacity (Juliano, 1971). Amylose content (AC) plays an important role in different rice cooking, sensory and processing properties (Bergman *et al.,* 2001). So there is a need to identify cultivars with targeted amylose content that suits the desired end-use quality characteristics.

Conventional methods of amylose content estimation by biochemical analysis is rapid and reproducible but environmental effects and genetical factors like dominance nature of inheritance may contribute for the error in the phenotypic measurement. The cooking quality of the rice grain is mainly determined by *Wx* gene locus as the amylose synthesis is mainly controlled by Waxy (*Wx*) gene locus in chromosome 6 which encode Granule Bound Starch Synthase (GBSS) enzyme (Smith *et al*., 1997). Polymorphic microsatellite marker RM 190 with $(CT)_n$ dinucleotide repeats at exon 1 in *Wx* gene locus was proved to give significant association with amylose content Bligh *et al*., 1995, Ayres *et al*., 1997 and Jayamani *et al*.,2007. Extensive studies on *Wx* gene

and amylose content have conducted on *japonica* genotypes. Intensive efforts in this direction in *indica* genotypes will be helpful in exploring the variability available in germplasm. The aim of this study was to detect polymorphisms in starch synthesizing genes among diverse landraces and cultivars of rice (*Oryza sativa* L.) and to determine the association with starch physicochemical properties.

MATERIALS AND METHODS

Eighty two genotypes of rice with largely different amylose contents and genetic backgrounds were selected including 75 local land races and few popular varieties along with two japonica cultivars. The selected material includes indica and japonica genotypes (Table 1). The seed materials were obtained from germplasm collection of Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The genotypes were studied for various quality parameters like amylose content (AC), grain length (GL), grain breadth (GB), LB ratio (LBR), alkali spreading value (ASV) and gelatinization temperature (GT). SSR marker RM 190 was employed to assess the genetic diversity among 82 rice genotypes for the genetic polymorphism of the *Wx* gene. The experiments for polymorphism analysis of the landraces were conducted at the Marker Assisted Selection laboratory, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, during 2011-2012. The biochemical analysis to detect amylose content in the genotypes was conducted at biochemical lab at Paddy Breeding Station (PBS), Coimbatore.

Table 1. List of genotypes

Variety	Variety	Variety
Sornavari	Thogai Samba	Vathra Iruppu
Kodaikulathan	Poongar	Kallurundai
Thattan Samba	Nallakonmani	Varappu Kudaichan
Red Sirumani	Uppu Molagai	Kuliadichan
Avasara Samba	Chetty Samba	Rasacadam
Saranga	Chittan Samba	Mattaikar
Peria Samba	Godavari Samba	Tadukkan
Earapalli Samba	Shenmolagai	Tep Tep
Manavari	Rangoon Samba	Jeeraga Samba
Malayalathan Samba	Karthi Samba	Thillainayagan
Aruputham Kuruvai	Val Samba	Co(R) 48
Vari Samba	Muthu Vellai	Co(R) 49
Panamara Samba	Sembalai	Poongar
Mikuruvai	Mohini Samba	Karthika Samba
Vellai Samba	Seevan Samba	Co(R) 50
Thooyala	Vadakathi Samba	GEB 24
Kodai	Koombalai	Jothi
Kalarkar	Palkachakka	Purple Puttu
Kallimadayan	Anaikomban	Hinottikari*
Thillai Nayagam	Karthigai Samba	Morebarekan*
Muzhi Karuppan	Koola Valai	Ptb 19
Vellai Chithreakar	Kattikar	Pavizham
Nootripathu	Ganthasala	
Norungan	Chinthamani	
Kallurundaikar	Varigarudan Samba	
Chivappam Chithraikar	Ponmani Samba	
Ponkambi Samba	Valanchennai	
Vellai Gundu Samba	Pokkali	
Mangam Samba	Kalvalai	
Thorai Samba	Vadivel	

*- belongs to japonica group

Total genomic DNA was extracted from the 20 days-old seedlings of 82 accessions using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). The dilutions were carried out by dissolving the genomic DNA in appropriate volume of TE buffer for the working sample. The polymorphism detection was performed for the genotypes using SSR primer RM190 (forward primer: 5'CTTTGTCTATCTCAAGACAC-3' and reverse primer: 5' TTGCAGATGTTCTTCCTGATG-3') (Ayres *et al*., 1997 and Teminykh *et al*., 2000) for amplifying genomic DNA fragment by polymerase chain reaction. Amplifications were performed on an AB PCR thermal cycler with a PCR profile of initial denaturation of 95°C for 4 minutes, denaturation of 94°C for 45 seconds, annealing of 55°C for 30 seconds, extension of 72°C for 1 minutes for a total of 35 cycles repetition from denaturing and a final extension of 72°C for 5 minutes. Poly acrylamide gel electrophoresis was performed to separate amplification products using a mega gel high throughput vertical unit (CBS Scientific, USA). Six percent polyacrylamide gels were used for better separation and visualization of PCR amplified microsatellite products and were allowed to run through the gel at 350V for 4 hour. Poly acrylamide gel staining was done with an improved silver staining protocol proposed by (Benbousa *et al*.,2006). After the staining process the PAGE gel on glass plate were visualized using an X-ray sheet illuminator with fluorescent lamp and the gel photographs were taken by Canon Powershot SX 200 camera with 12X optical zoom.

Amylose content was determined for all the 82 genotypes using the following modified IRRI

method. For this purpose 500g of rice grains were dehusked and the polished grains were grinded using the coffee grinder. For the analysis 100g of finely powdered rice flour was weighed as test sample and transferred to volumetric flasks. One ml of 75% ethanol and 9.0 ml of NaOH (1M) was added to the sample and was kept overnight for digestion. A blank was also prepared simultaneously. The dissolved sample was made upto 100 ml with distilled water. Aliquote (0.5 ml) was transferred to two test tubes and 0.10 ml of acetic acid and 0.20 ml of iodine solution (0.2%) was added to the test tube. The volume was made upto 10.00 ml by adding distilled water and the solution was mixed in vortex meter. Spectro-photometric quantification of absorbance was performed at 720 nm. Two determinations were made on separate test portions taken from same sample in each of the two replications. The apparent amylose content was calculated based on a rice standard curve and reported as percentage on dry weight basis.

The rice grain morphological and quality parameters like grain length, grain breadth, LB ratio, grain colour, alkali spreading value and gelatinisation temperature were scored and recorded based on Standard Evaluation System (SES), IRRI, 2002. The time required for cooking of milled rice is determined by gelatinization temperature or GT. Gelatinization temperature (GT) was obtained by measuring the alkali spreading value (ASV) of the milled grains and is considered to be inversely related to GT. The method as suggested by Little *et al*., 1958 was followed. The method involves incubating six intact grains of milled rice in 10 ml of 1.7% KOH at 30^{O}C for 23 h and the degree of spreading is measured using a seven-point scale.

Based on amylose content, the genotypes were classified into different amylose groups (SES, 2002) as low amylose (10-20% amylose), intermediate amylose (20-24% amylose) and high amylose (>25% amylose). Based on alkali spreading value, the genotypes were grouped(SES, 2002) as follows: (1) grain not affected; (2) grain swollen; (3) grain swollen, collar incomplete and narrow; (4) grain swollen, collar complete and wide; (5) grain split or segmented, collar complete and wide; (6) grain dispersed, merging with collar; and (7), grain completely dispersed and intermingled.

The employment of SSR markers in genetic diversity analysis also helped in grouping the genotypes based on amylose content level. The marker data was subjected to cluster analysis and dendrogram was drawn by using the software NTSYSpc ver.2.2. The genetic association with 82 genotypes were evaluated using Jaccard's similarity coefficient and clustered with unweighted pair group method of arithametical averages (UPGMA) analysis (Rohlf,2002).

RESULTS AND DISCUSSION

More than half of the world's population uses rice as a source of carbohydrate intake every day. Improvement in plant breeding techniques has greatly increased the yield in rice cultivation, but grain quality of developed varieties to be improved further to meet various needs. Improving grain quality is thus essential to rice consumers (Tian *et al*., 2009). Major part of rice grain is composed of starch and its characteristics determine cooking and consumption qualities and the quality of various products of rice. The starch reserves of rice grains generally contain 17-30% amylose, which plays an important role in palatability and processing qualities (Ramesh *et al*.,1999 and Zhou *et al*.,2003). In the present investigation starch synthesizing genes were detected among diverse indica landraces and cultivars of rice (*Oryza sativa* L.) and determined their correlation with amylose content and grain traits. The study involved biochemical analysis of amylose content in 82 genotypes in rice along with molecular analysis of detection of polymorphism in the waxy gene locus using microsatellite markers.

Rice grain with low amylose content will be tender, cohesive, glossy and sticky in nature and the high amylose content can cause firm fluffy and separate grains of cooked rice. According to the International Rice Research Institute, the non-glutinous rice represents 'low' (<19%), 'intermediate' (20-25%) and 'high' (>25%) amylose strains. Based on mean values of amylose content, the 82 genotypes were classified and given in Table 2. The lines under this study were not coming under the first two categories of waxy or very low amylose but were under intermediate to high amylose types. The amylose content ranged from 14.22% (*Ganthasala*) to 33.52% (*Vadivel*). The genotypes were studied for various physio chemical properties like grain length, grain breadth, LB ratio, grain

Table 2. Classification of genotypes used in the study based on amylose content.

Low amylose (10-20%)		Intermediate amylose (20-24%)		High amylose (>24%)	
Variety	Amylose Content	Variety	Amylose Content	Variety	Amylose Content
Ganthasala	14.22	Thattan Samba	20.15	Kodai Kulathan	24.38
Vadakathi Samba	14.33	Poongar	20.25	Moorebarekan	24.39
Thogai Samba	14.52	Co(R) 48	20.39	Vellai Chithrakar	25.10
Karthigai Samba	15.11	Thorai Samba	20.55	Thillainayagan	25.13
Mohini Samba	15.71	Poongar	20.56	Mikuruvai	25.17
Anaikomban	16.88	Muthu Vellai	20.60	Vari Samba	25.30
Seevan Samba	17.07	Muzhi Karuppan	20.61	Sornavari	25.86
Nootripathu	17.16	Co(R) 50	20.66	Varappu Kudaichan	25.93
Chittan Samba	17.33	Pokkali	20.72	Rasacadam	26.28
Koombalai	17.63	Saranga	20.81	Chivappam Chithraikar	26.35
Rangoon Samba	17.78	Thooyala	20.95	Malayalathan Samba	26.94
Kodaikulathan	17.87	Godavari Samba	20.99	Vellai Samba	27.43
Sembalai	18.12	Shenmolagai	21.05	Vathra Iruppu	27.88
Chetty Samba	18.17	Avasara Samba	21.07	Kallurundaikar	29.26
Nallakonmani	18.26	Red Sirumani	21.08	Kalarkar	29.55
Ponkambi Samba	18.41	Manavari	21.12	Valanchennai	29.64
Vellai Gundu Samba	18.43	Palkachakka	21.19	Kallurundai	30.46
Uppu Molagai	18.54	Koola Valai	21.34	Pavizham	30.47
Val Samba	18.57	Norungam	21.59	PTB 19	30.94
Purple Puttu	18.94	Jeeraga Samba	21.67	Thillai Nayagam	31.99
Peria Samba	18.96	Hinottikari	21.68	Vadivel	33.52
Tadukkan	18.99	Jothi	21.77		
Karthika Samba	19.12	Earapalli Samba	21.85		
Mattaikar	19.41	Panamara Samba	22.15		
GEB 24	19.44	Karthi Samba	22.45		
Chinthamani	19.49	Co(R) 49	22.55		
Tep Tep	19.49	Mangam Samba	22.82		
Kallimadayan	19.85	Ponmani Samba	23.02		
Kattikar	19.94	Aruputham Kuruvai	23.34		
		Kuliadichan	23.34		
		Kalvalai	23.55		
		Varigarudan Samba	23.65		

colour, alkali spreading value and gelatinisation temperature (Table 3). Correlation analysis among these traits revealed highly significant correlation between grain length and grain breadth with LBR ratio and Gelatinisation temperature was showing significant negative association with alkali spreading value (Table 4) and same results were reported by Lapitan *et al*., 2009,Umadevi *et al*.,2010 and Ravi *et al*., 2012.

The SSR markers employed in genetic diversity analysis was found to be useful in grouping the genotypes based on amylose content level. The SSR primer, RM190 showed 48.95% correlation with phenotypical variation (Fig.1). Significant correlation was not identified by RM 190 genotypic data with other grain quality traits. The marker data and phenotypical data were subjected to cluster analysis and using the software NTSYSpc ver.2.2. The dendrogram clustering showed that the 82 genotypes can be clustered into three major groups at 60% level of genetic similarity based allelic information of RM190 (Fig.2). The 82 genotypes were evaluated using Jaccard's similarity coefficient and clustered with unweighted pair group method of arithametical averages (UPGMA) analysis based on their amylose content. The clusters contain genotypes which are similar in the amylose content levels upto 50% of similarity (Fig.3). The difference in amylose content levels of genotypes with same allelic

data may be due to environmental effects and due to influence of other genes in the starch synthesis pathway as reported by Juliano and Pascual, 1980. Amylose content measurement by calorimetry method also detects various levels of long chain amylopectin (Takeda *et al*.,1987). Amylose content is also controlled by partial or complete dominance which influences phenotypic measurement Pooni *et al*., 1993. Amylose content variation up to six percent for a given cultivar can be caused by environmental effects (Juliano and Pascual, 1980 and Asaoka *et al*., 1985).

Table 3. Different physiochemical properties of landraces used in the study

Sl. No.	AC	GL	GB	LB R	ASV	GT	GC
Lr1	25.86	5.00	2.00	2.50	2.00	5.00	1.00
Lr2	17.87	7.00	2.00	3.50	4.00	3.00	1.00
Lr3	20.15	6.10	1.50	4.07	3.00	3.00	1.00
Lr4	21.08	5.00	3.00	1.67	2.00	5.00	4.00
Lr5	21.07	5.00	2.00	2.50	2.00	5.00	4.00
Lr6	20.81	4.00	2.50	1.60	2.00	5.00	1.00
Lr7	18.96	5.50	1.50	3.67	2.00	5.00	2.00
Lr8	21.85	5.00	2.00	2.50	1.00	5.00	2.00
Lr9	21.12	5.50	2.00	2.75	1.00	5.00	5.00
Lr10	26.94	5.30	2.00	2.65	1.00	5.00	5.00
Lr11	23.34	5.50	2.00	2.75	1.00	5.00	2.00
Lr12	25.3	5.00	1.50	3.33	2.00	5.00	3.00
Lr13	22.15	6.00	1.50	4.00	1.00	5.00	1.00
Lr14	25.17	5.00	2.00	2.50	1.00	5.00	6.00
Lr15	27.43	5.20	2.00	2.60	1.00	5.00	1.00
Lr16	20.95	5.00	2.00	2.50	2.00	5.00	1.00
Lr17	24.38	6.50	1.50	4.33	1.00	5.00	2.00
Lr18	29.55	5.50	2.00	2.75	1.00	5.00	2.00
Lr19	19.85	5.00	2.00	2.50	1.00	5.00	2.00
Lr20	31.99	7.00	2.00	3.50	1.00	5.00	1.00
Lr21	20.61	5.50	2.00	2.75	1.00	5.00	1.00
Lr22	25.1	5.00	1.50	3.33	2.00	5.00	1.00
Lr23	17.16	5.10	2.50	2.04	2.00	5.00	4.00
Lr24	21.59	5.50	2.50	2.20	3.00	3.00	4.00
Lr25	29.26	6.50	1.50	4.33	1.00	5.00	1.00
Lr26	26.35	5.00	2.50	2.00	2.00	5.00	3.00
Lr27	18.41	5.30	2.00	2.65	2.00	5.00	1.00
Lr28	18.43	4.50	3.00	1.50	1.00	5.00	1.00
Lr29	22.82	4.75	2.00	2.38	1.00	5.00	1.00
Lr30	20.55	5.00	2.00	2.50	1.00	5.00	1.00
Lr31	14.52	5.50	1.50	3.67	1.00	5.00	1.00
Lr32	20.56	6.50	2.00	3.25	2.00	5.00	4.00
Lr33	18.26	5.00	2.00	2.50	1.00	5.00	1.00
Lr34	18.54	5.50	1.75	3.14	1.00	5.00	1.00
Lr35	18.17	4.50	2.30	1.96	2.00	5.00	4.00
Lr36	17.33	6.00	2.00	3.00	2.00	5.00	7.00
Lr37	20.99	5.00	2.00	2.50	2.00	5.00	1.00
Lr38	21.05	5.50	2.50	2.20	2.00	5.00	4.00
Lr39	17.78	4.50	2.00	2.25	2.00	5.00	4.00
Lr40	22.45	5.50	1.50	3.67	1.00	5.00	2.00
Lr41	18.57	6.00	2.00	3.00	2.00	5.00	2.00
Lr42	20.6	5.00	2.00	2.50	2.00	5.00	1.00
Lr43	18.12	5.50	2.00	2.75	2.00	5.00	2.00
Lr44	15.71	5.50	2.00	2.75	2.00	5.00	1.00
Lr45	17.07	5.00	2.00	2.50	2.00	5.00	2.00
Lr46	14.33	6.00	1.50	4.00	2.00	5.00	6.00
Lr47	17.63	5.50	2.00	2.75	1.00	5.00	1.00
Lr48	21.19	5.00	2.00	2.50	1.00	5.00	1.00
Lr49	16.88	4.00	2.00	2.00	1.00	5.00	1.00
Lr50	15.11	5.50	2.50	2.20	4.00	3.00	1.00
Lr51	21.34	6.00	2.00	3.00	3.00	3.00	4.00
Lr52	19.94	5.20	2.00	2.60	2.00	5.00	2.00
Lr53	14.22	5.20	2.00	2.60	2.00	5.00	2.00
Lr54	19.49	4.50	1.50	3.00	2.00	5.00	1.00
Lr55	23.65	5.50	2.50	2.20	2.00	5.00	4.00
Lr56	23.02	4.75	2.50	1.90	2.00	5.00	2.00
Lr57	29.64	4.80	2.20	2.18	2.00	5.00	5.00
Lr58	20.72	7.00	2.00	3.50	1.00	5.00	3.00
Lr59	23.55	6.00	2.00	3.00	3.00	3.00	6.00
Lr60	33.52	6.50	2.00	3.25	4.00	3.00	2.00
Lr61	27.88	6.50	2.00	3.25	4.00	3.00	2.00
Lr62	30.46	5.00	2.20	2.27	2.00	5.00	1.00
Lr63	25.93	4.50	3.00	1.50	3.00	3.00	5.00
Lr64	23.34	6.00	3.00	2.00	2.00	5.00	5.00
Lr65	26.28	3.75	1.75	2.14	1.00	5.00	2.00
Lr66	29.8	5.75	2.00	2.88	2.00	5.00	1.00
Lr67	18.99	5.00	2.00	2.50	2.00	5.00	2.00
Lr68	19.49	5.50	1.75	3.14	2.00	5.00	1.00
Lr69	21.67	3.50	1.25	2.80	1.00	5.00	2.00
Lr70	25.13	7.50	2.00	3.75	6.00	1.00	2.00
Lr71	20.39	6.00	2.00	3.00	2.00	5.00	2.00
Lr72	22.55	5.00	1.50	3.33	7.00	1.00	1.00
Lr73	20.25	6.50	2.00	3.25	3.00	3.00	5.00
Lr74	19.12	5.20	2.30	2.26	2.00	5.00	1.00
Lr75	20.66	5.75	2.00	2.88	2.00	5.00	2.00
Lr76	19.44	5.00	1.75	2.86	5.00	3.00	1.00
Lr77	21.77	5.50	2.00	2.75	6.00	1.00	5.00
Lr78	18.94	5.00	2.50	2.00	2.00	5.00	7.00
Lr79	21.68	5.50	2.00	2.75	5.00	3.00	1.00
Lr80	24.39	6.00	2.20	2.73	3.00	3.00	4.00
Lr81	30.94	5.00	2.30	2.17	2.00	5.00	5.00
Lr82	30.47	5.10	2.00	2.55	2.00	5.00	6.00
Mean	22.92	5.39	2.03	2.74	2.10	4.54	2.51
Min.	12.06	3.50	1.25	1.50	1.00	1.00	1.00
Max.	33.60	7.50	3.00	4.33	7.00	5.00	7.00
Var.	16.91	0.53	0.13	0.39	1.52	1.02	3.02
SD	4.11	0.73	0.36	0.62	1.23	1.01	1.74
SE	0.45	0.08	0.04	0.07	0.14	0.11	0.19

AC- Amylose content, GL-grain length, GB- grain breadth, LBR-length-breadth ratio, ASV-alkalispreading value,GT-gelatinisation temperature, GC- grain colour

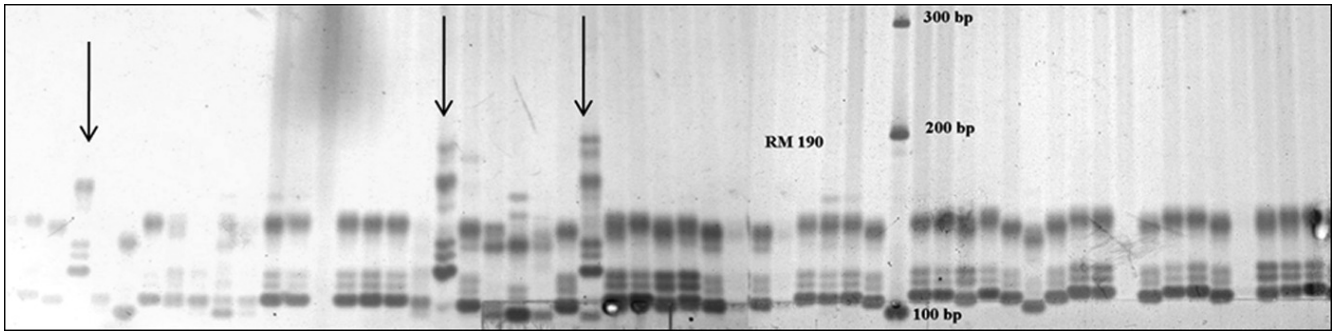

Fig. 1. Polymorphism among different land races for amylose content by the marker RM190 by Poly Acrylamide Gel Electrophoresis(PAGE)

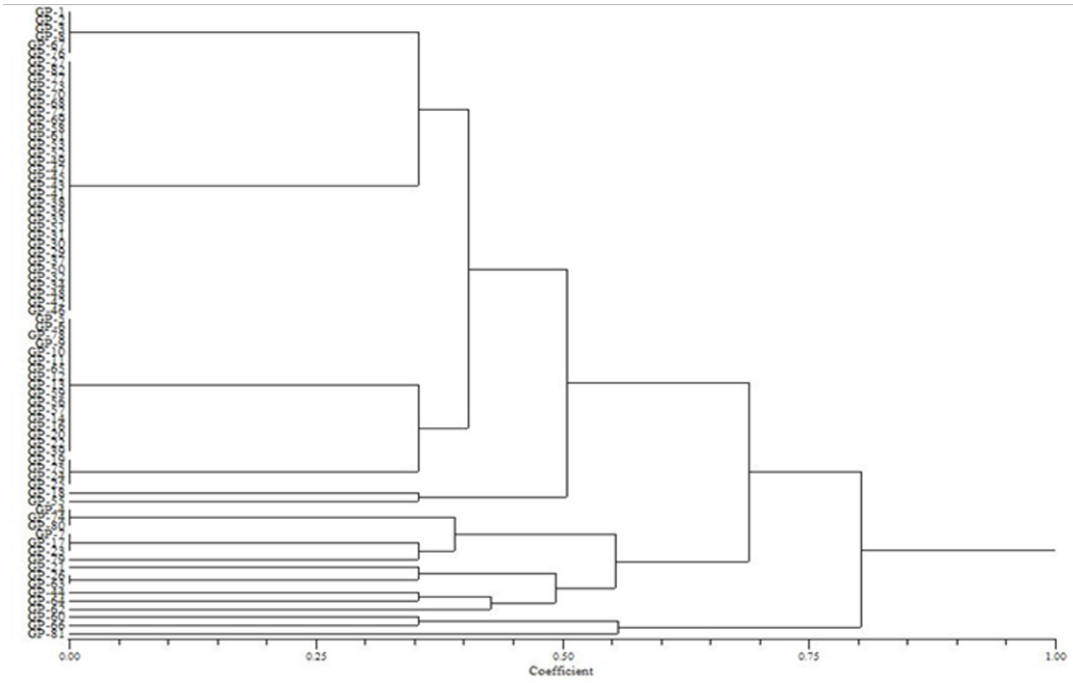

Fig. 2. UPGMA dendrogram showing clustering of 82 genotypes of rice based on genotypic data

Table 4. Correlation between different physiochemical properties of landraces used in the study

	AC	GL	GB	LB R	ASV	GT	GC
AC	1.00						
GL	0.16	1.00					
GB	0.05	-0.13	1.00				
LB R	0.04	0.67**	-0.79**	1.00			
ASV	0.00	0.26*	0.04	0.11	1.00		
GT	-0.08	-0.34*	0.00	-0.20	-0.90**	1.00	
GC	-0.08	0.07	0.31*	-0.18	0.06	-0.07	1.00

Marked correlations are significant *at p < .05000, **at p < 0.01000, AC- Amylose content, GL-grain length, GB- grain breadth, LBR- length-breadth ratio, ASV-alkalispreading value,GT-gelatinisation temperature, GC- grain colour

Three genotypes *viz.,* PTB 19, Mattaikar and Vadivel formed the first cluster and which were under high amylose group based on biochemical study. The two japonica varieties came under second cluster with those of intermediate amylose content but Moroborekan showed high amylose content in biochemical analysis. Microsatellite marker (SSR) RM 190 identified 66 local land races in the study under third cluster and has probably originated from closely related ancestors and possesses high degree of genetic similarity. Microsatellite marker (SSR) RM 190 is useful in genotypic screening of germplasm lines for desired amylose content. The genotypes identified in the different amylose groups can be used in further quality

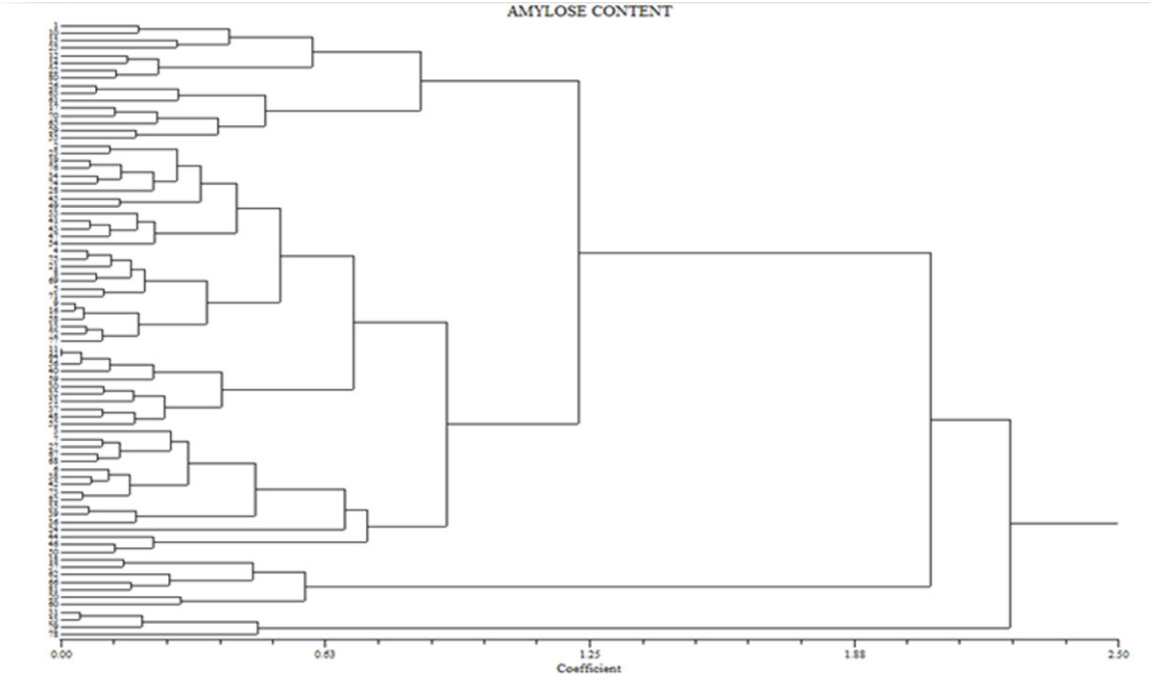

Fig. 3. UPGMA dendrogram showing clustering of 82 genotypes of rice based on phenotypic data

improvement programmes as donor sources for development of various end use products. Improvement in plant breeding techniques has greatly increased the yield in rice cultivation, but there is a need to improve grain quality of developed varieties to meet the consumer preference and wide acceptance among the farmers.

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