MOLECULAR ANALYSIS OF GLUTINOUS PIGMENTED RICE GERMPLASM THROUGH THE AID OF HIGHLY POLYMORPHIC MARKERS

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ABSTRACT

The challenge for making food for less is to make it healthier and with value-added traits i.e. glutinous and pigmented. It is important in any crop breeding to increase heterosis by combining distant genotypes and this could be achieved through the use of molecular markers. This study aimed to assess the genetic distance of 83 entries with 71 elite lines consisted of pigmented, glutinous and pigmented- glutinous rice, 9 parent lines and one check variety, NSIC Rc19. Preliminary survey of 300 genome-wide microsatellite markers led to 17 highly polymorphic markers. Overall diversity among the entries is 48% which is expected among sister lines. A 100% similarity was observed for two pairs of elite lines. Dendrogram generated based on UPGMA formed two major clusters with one elite line Line21 and Traditional4 and Traditional7 in one cluster while the rest of the entries formed the other cluster. Without markers, the breeding of special purpose rice including combination of pigmented and glutinous rice varieties would take longer but with highly polymorphic microsatellite markers could accelerate the development of new special purpose rice varieties.

INTRODUCTION

Rice is the staple food for over 3 billion people, comprising over half of the world's population. Philippines is one of the recognized center origin of rice endowed with remarkably rich rice diversity. Food Agricultural Organization (2015) reported that Philippines is the 9th largest producer of rice worldwide. Aside from white rice commonly produced and consumed by most of the Filipinos, existence of other types of rice have been reported especially glutinous-pigmented (red, brown, purple, black) rice that exhibit and shows high phenolic, anthocyanin and antioxidant contents (Phonsakhan and Kong-Ngern, 2015). This form of rice is a combination of glutinous and pigmented rice in which glutinous rice have no or have negligible amounts of amylose and high amounts of amylopectin that turns up as a paste or gel when cooked (Pathak et al. 2016), while, pigmented or colored rice has been preferred by most of individual due to its special features in medicinal value and exclusive taste as compared to white rice (Patel et al. 2014). Hence, glutinous-pigmented rice variety has gained a lot of attention as raw materials for production of health food supplements in human consumption (Phonsakhan and Kong-Ngern, 2015).

The knowledge of the extent of diversity in rice is essential for rice crop improvement as it helps breeders in deciding suitable breeding strategies for their future improvement (Singh et al. 2016). It is generally thought that continuous selection among the crosses of genetically related cultivars has led to a narrowing of the genetic base of the crops particularly in rice. Yield, quality characters and tolerance to biotic and abiotic stresses are major objectives of varietal development (Nachimuthu et al. 2015). Reliable method of fingerprinting is required for identification of these varieties, as well as to study the genetic relationship among different cultivars (Kibria et al 2009 and Sivaranjani et al 2010). Nowadays, SSR marker have proven to be a marker of choice for evaluating genetic diversity and relationship among plants in studying rice germplasm for either conservation or utilization, marker assisted selection

breeding, cultivar identification, hybrid purity analysis and gene mapping studies (Tu et al. 2007; Sharma et al. 2007; Rani and Adilakshin, 2011; Rajendrakumar et al. 2009; and Sarao et al. 2010). Consequently, SSR markers are co-dominant, distributed throughout the genome, highly reproducible, variable, reliable, easily scorable, abundant and multiallelic in nature (Salgotra et al. 2015). Thus, many researchers have been used SSR markers in characterizing the diversity of rice varieties (Das et al. 2013; Jin et al. 2010; and Sow et al. 2014). The present study was undertaken with the aim to assess the genetic diversity of 71 elite lines consisted of pigmented, glutinous and pigmented-glutinous rice germplasm along with 9 parent lines and 1 check variety using Simple Sequence Repeat (SSR) markers.

MATERIALS AND METHODS

Experimental material

The study was designed to evaluate the genetic analysis of sisters and parent lines of glutinous-pigmented rice genotypes. Three hundred microsatellite primer pairs were used for the amplification of DNA fragments. Twenty microsatellite markers were selected from the original screening for analyzing the variability of 83 glutinous pigmented rice genotypes. The list of selected genotypes is given in Table 1.

DNA Extraction

Young leaves were harvested and placed in glassine bags, transported in ice and stores at -20°C. Plant DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) method as modified by Saghai-Maroof et al. (1994). The DNA extracted was diluted in 100ul of TE Buffer (Tris EDTA buffer–10 mM Tris HCl, 1 mM EDTA, pH 8.0). The tubes were left for few hours to allow DNA to dissolve. Quantity of DNA was determined by spectrophotometer and quality was determined using agarose gel electrophoresis.

PCR Analysis and Gel Electrophoresis

A set of twenty SSR primers were used and the sequence of primers was obtained from website http://www.gramene.org/microsat.tex (Table 2). The PCR reaction was carried out using 0.044ul of 500units *Taq* polymerase with 1ul of 5X PCR buffer, 0.15ul 25mM $MgCl_2$, 0.5ul 5mM dNTPs, 0.4ul of 10mM primer forward, 0.4ul of 10mM of primer reverse and 1.3ul of DNA template. Thermal cycle program was set as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation step at 94°C for 1 min, annealing step at 60°C for 1 min, elongation step at 72°C for 2 min and final extension at 72°C for 7 min. The SSR-PCR products were analyzed on 8% polyacrylamide gel using DNA ladder (1kb plus) and were visualized by staining with gel red.

Data Analysis

The amplified products from SSR marker analysis were scored quantitatively for presence (1) or absence (0) for each marker allele-genotype combination. The molecular weights of PCR products were calculated based on ladder of known molecular weight of 1kb plus. The distance matrices were used for cluster analysis. The similarity and distance matrix were thus generated and dendogram was constructed using UPGMA (Unweighted Pair Group Method using Arithmetic Averages) available in NTSYSpc 2.21p (Rohlf, 2000). Using polymorphic bands generated by SSR markers, the graphical representation of DNA fingerprint was developed.

RESULTS AND DISUSSION

The present study evaluated the genetic diversity of 83 glutinous-pigmented rice genotypes consists of 71 elite lines and 9 parent lines. Genetic diversity is needed for developing ideal, desired crop varieties for present and future needs. In this study, 300 SSR

markers were screened, out of which 16 SSR markers were found to be polymorphic and suitable for diversity analysis. The use of SSR for rice diversity study is very essential as it provides accurate and unbiased assessment and reveals in-depth information on the genetic diversity of germplasm materials (Ahmad et. al. 2015). Also, microsatellite (SSR) markers are the PCR based markers that have been developed in many plant species; they have an advantage of being multi allelic, highly polymorphic and codominant. The use of SSR markers to investigate genotypic variations among different rice cultivars was previously reported by some researchers (Singh et al. 2004 Joshi and Behera 2006).

SSR Polymorphism

From 300 SSR primers used to generate marker profiles among which 16 succeeded in amplifying the 83 glutinous-pigmented rice samples, producing a total of 50 alleles per locus. These 15 primer pairs are well spread on all the chromosome except 2 and 4. The majority of the SSR loci differed appreciably in the number of alleles. The number of alleles per locus generated by each primer varied from 3 to 4, with an average of 3.33 alleles. The number of alleles indicates the richness of the population. The gel picture showing banding pattern of 83 glutinous pigmented rice genotypes using RM6051 marker is given in Figure 1. Lane 1-11, 14-18 and 20 showed 350bp/390bp while lane 12-13 and 19 showed 390bp/400bp.The low number of alleles was usually obtained from a collection of breeding lines and closely related cultivars such as those used by Zeng et. al. (2004). Also, high number of alleles was expected to be found when large number of landraces from a wide range of geographical origins is included in the study (Brondani et. al. 2016).

These mean alleles obtained in the study were comparable with the result reported by (Sajib et. al. 2012) detecting 3.3 alleles per SSR locus who used 12 aromatic rice genotypes investigated using SSR markers. Also, this were also comparable with the result of (Ji et. al.

2007) detecting a mean of 3 alleles per locus in colored rice lines using SSR markers and in 42 colored upland rice genotypes reported by (Ahmad et. al. 2015) with 3.24 mean of allele per locus. In contrast, the mean value obtained from our study is somewhat lower than the results observed in Vietnamese colored rice accessions with an average of 7.1 alleles per locus using 40 SSR markers. Furthermore, highest allele number of 6 using RM334 marker was also recorded in colored upland rice germplasm (Ahmad et. al. 2015), 4 alleles detected in glutinous pigmented rice germplasm and 7 alleles found in aromatic landraces which was comparable in the present study showing 5 alleles. However, marker RM334 shows 3 (Islam et. al. 2018), 4 (Anandan et. al. 2016) and 2 (Singh et al. 2015) alleles per locus in rice germplasm which is lower than the number of alleles recorded in the present study. Additionaly, lowest number of alleles (3) was recorded in RM328, RM259 and RM114 which is lower than the findings of Lapitan et. al. (2007), Ashraf et. al. (2016) and Ahmad et. al. 2015. These findings implying thereby that the same primers provide different information content depending on genotypes under study.

The observed genetic diversity index varied from 0.26 to 0.71, averaging 0.46 with a genetic similarity (GS) coefficient of 0.52, reflecting a moderate level of genetic diversity. The highest genetic diversity (0.71) was recorded in RM334 and the lowest genetic diversity (0.26) was recorded in locus RM328. This result was comparable with the findings of (Islam et. al. 2016) using 50 red germplasm in Bangladesh with a gene diversity of 0.05 to 0.68, averaging 0.35 which also indicate moderate level of diversity. This was also comparable with the previously reported by (Hossain et. al. 2007) in aromatic rice using SSR markers with genetic diversity ranging from 0.28 to 0.7917, averaging 0.5570, indicating moderate level of diversity.

Polymorphic information content (PIC) value is a reflection of allelic diversity and frequency among the genotypes. In the present study, highest polymorphism content (PIC) of 0.65 for the marker RM334 and lowest PIC value of 0.24 was recorded for the marker RM328,

averaging 0.40. Three markers were highly informative, 9 markers were moderately informative and 3 markers were slightly informative. There was correlation between the PIC values and the number of alleles at SSR loci. Comparatively, moderate mean PIC value could be due to: limited number of genotypes that are very well acclimatized to the local environment, less differences in the contribution of the marker DNA regions, genepool with narrow genetic base, lastly, less mutation rate in the di/tri repeat (Giarrocco et. al. 2007).This results was lower than PIC average value reported by (Hue et. al. 2018) of local colored rice variety with PIC values ranged from 0.41 to 0.89, averaging 0.74 using 40 SSR markers. The maximum PIC value of 0.65 in our study was recorded for SSR RM334 which was also shown in the study of Ahmad et. al. 2015 (0.74), Islam et. al. 2015 (0.52) and Anandan et. al. 2016 (0.80). Similarly, RM162 which has been previously reported to show PIC value of 0.95 (Pathank et. al. 2016) and 0.65 (Ashraf et. al. 2016) while RM514 has shown 0.55 (Ahmad et. al. 2015) was highly informative and PIC value in the present study was 0.6370 and 0.6415, respectively. This indicated that that these three primers (RM334, RM162 and RM514) are effective and useful in determining the genetic difference among the 100 rice genotypes of the glutinous pigmented rice germplasm and to study the phylogenetic relationships between them.

Cluster Analysis

Genetic similarity values among the rice cultivars used led to the construction of a dendogram presented in Figure 2. At a 52% level of similarity, the UPGMA cluster diagram showed 2 groups with additional sub-clusters with each group. This dendogram revealed that the cultivars derived from a genetically similar type clustered together. Group 1 corresponded to the glutinous pigmented sister lines, whereas Group 2 comprised of variety PR41622-B-B-37-4-3-1, Ominio and Venere. Group 1 comprised the 9 sub-cluster wherein sub-cluster 1 consists the majority of the glutinous, pigmented and glutinous-pigmented rice lines with 40 entries and 8, 3, 3, 2, 7, 1, 14, 1, 1, 1, 1 in subclusters 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, respectively.

All these cultivars in the sub-cluster 1 exhibited a waxy to very low amylose content and some are intermediate and high amylose content. In addition, parent lines such as Red Rice, Red Rice and Ballatini were grouped in sub-cluster 6 while black rice from Ilo-Ilo and Thailand were grouped in sub-cluster 8 which indicate that with the same caryopsis color grouped together.

Highest similarity of 100% was observed for three pairs of elite lines PR46443-B-B-4-1-2-1 (46) and PR46443-B-B-4-1-4-1 (50), PR46443-B-B-4-2-1-2 (53) and PR46443-B-B-4-2-3-1 (54), PR41621-B-B-19-3-2-2 (74) and PR41621-B-B-19-2-3-2 (75) which is expected among sister lines. However, lowest similarity of 40% was observed between Ominio (36) and PR41621-B-B-10-1-3-1 (21) while most of the breeding lines such as: PR44015-B-35-1, PR41621-B-B-19-2-2-1, PR37045-B-6-1-1-1-1-2-1-1-2, PR 37042-B-1-1-2-6 (BL/G), PR46443-B-B-4-1-4-2, PR46443-B-B-4-3-2-1 and PR46443-B-B-4-3-2-2 were distant to the parent line “Venere”. In this study, the larger range of similarity values for genotypes revealed by SSR markers provides greater confidence for the assessments of genetic diversity and relationships, which can be used in future breeding program.

CONCLUSION

The study showed the existence of the essential variability of 71 elite lines and 9 parent lines of glutinous-pigmented rice variety that could be used in crop improvement through the use of 16 highly polymorphic markers. Therefore, without markers, the breeding of special purpose rice including combination of pigmented and glutinous rice varieties would have high percentage of genetically similar new varieties but with highly polymorphic microsatellite markers, this could accelerate the development of diverse new glutinous pigmented rice varieties.

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Table 1. List of rice genotypes used in the study.

No.	Entry Code	Designation	No.	Entry Code	Designation	No.	Entry Code	Designation	No.	Entry Code	Designation	No.	Entry Code	Designation
1	56	Modern1	21	86	Line19	41	135	Line34	61	159	Line52	81	70	Line70
2	58	Line1	22	87	Line20	42	136	Line35	62	160	Line53	82	71	Line71
3	59	Line2	23	88	Line21	43	137	Line36	63	161	Line54	83	72	Modern3
4	60	Line3	24	89	Traditional2	44	142	Traditional6	64	162	Line55			
5	63	Line4	25	90	Traditional3	45	143	Traditional7	65	163	Line56			
6	65	Line5	26	91	Line22	46	144	Line37	66	164	Line57			
7	66	Line6	27	92	Line23	47	145	Line38	67	165	Line58			
8	73	Line7	28	93	Line24	48	146	Line39	68	166	Line59			
9	74	Line8	29	94	Line25	49	147	Line40	69	167	Line60			
10	75	Line9	30	95	Line26	50	148	Line41	70	181	Traditional8			
11	76	Line10	31	96	Line27	51	149	Line42	71	186	Line61			
12	77	Line11	32	97	Line28	52	150	Line43	72	61	Line62			
13	78	Line12	33	98	Line29	53	151	Line44	73	62	Line63			
14	79	Line13	34	99	Line30	54	152	Line45	74	63	Line64			
15	80	Line14	35	101	Foreign1	55	153	Line46	75	64	Line65			
16	81	Line15	36	102	Traditional4	56	154	Line47	76	65	Line66			
17	82	Traditional1	37	127	Line31	57	155	Line48	77	66	Line67			
18	83	Line16	38	132	Line32	58	156	Line49	78	67	Modern2			
19	84	Line17	39	133	Line33	59	157	Line50	79	68	Line68			
20	85	Line18	40	134	Traditional5	60	158	Line51	80	69	Line69			

Table 2. Genetic index of 24 SSR markers used for genetic diversity of 86 pigmented rice germplasm.

Marker	Major Allele Freq.	Genotype No.	Allele No.	Gene Diversity	Heterozygosity	PIC
RM328	0.86	3	3	0.26	0.00	0.24
RM259	0.84	3	3	0.28	0.00	0.26
RM114	0.84	4	3	0.29	0.01	0.27
RM6051	0.77	3	3	0.36	0.00	0.31
RM171	0.70	4	3	0.43	0.02	0.36
RM495	0.77	4	4	0.40	0.19	0.37
RM44	0.75	4	4	0.42	0.00	0.39
RM25121	0.50	4	3	0.51	0.90	0.39
RM452	0.50	3	3	0.51	0.98	0.39
RM536	0.67	3	3	0.48	0.00	0.41
RM271	0.67	3	3	0.48	0.00	0.42
RM447	0.56	4	3	0.56	0.01	0.49
RM162	0.43	3	4	0.62	0.87	0.54
RM514	0.48	4	4	0.64	0.96	0.58
RM334	0.36	5	4	0.71	0.01	0.65
Mean	**0.65**	**3.60**	**3.33**	**0.46**	**0.26**	**0.40**

Table 3. Allele variation for SSR loci across 83 pigmented rice genotypes

Markers	Chr no.	Expected size	SSR motifs
RM6051	9	136	(CCG)10
RM328	9	172	(CAT)5
RM25934	10	110	(CAT)7
RM25121	10	133	(TCTA)5
RM495	1	159	(CTG)7
RM259	1	162	(CT)17
RM452	2	209	(GTC)9
RM514	3	259	(AC)12
RM44	8	99	(GA)16
RM271	10	101	(GA)26
RM334	5	182	(CTT)20
RM114	3	191	(GA)7
RM171	10	328	(GATG)5
RM162	6	229	(AC)20
RM447	8	111	(CTT)8
RM536	11	243	(CT)16

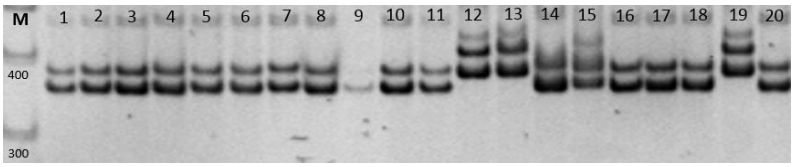

Figure 1. Representative gel showing polymorphic banding patterns. Amplification of genomic DNA from different rice varieties using SSR marker RM6051. Lane 'M' – molecular weight, lanes 1 to 20 – glutinous pigmented rice germplasm used in the study.

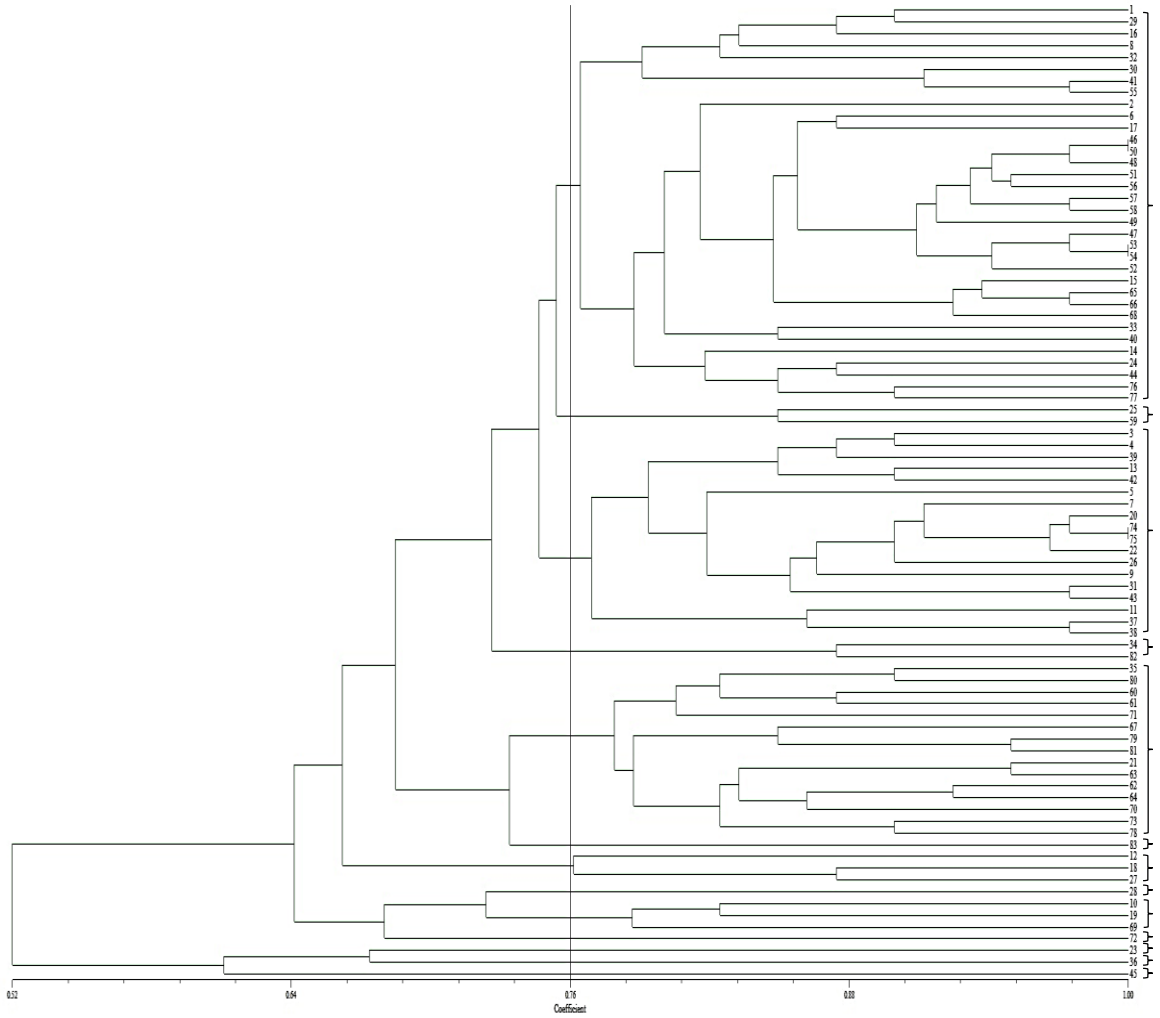

Figure 2. Dendrogram of 83 glutinous pigmented rice derived from UPGMA cluster analysis using NTSYS v2.0 based on 20 polymorphic SSR markers.