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ESTIMATION OF GENETIC DIVERSITY AND IDENTIFICATION OF POTENTIAL RICE LINES FOR TWO-LINE HYBRID BASED ON MICROSATELLITE MARKER AND PHENOTYPIC TRAIT ANALYSES

NUR AZREEN, M., SIJU, S., BHUIYAN, M.A.R. and WICKNESWARI, R.

Faculty of Science and Technology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia
**E-mail: atiqbhuiyan@ukm.edu.my*

ABSTRACT

The genetic relatedness among 31 advanced breeding lines derived from crosses between *O. rufipogon* and MR219, three Malaysian rice varieties (MR219, MR253 and MR263) and two thermosensitive genic male sterile (TGMS) lines (IR73827-23-26-15-7S and IR77271–42–5–4–36S) were determined using different microsatellite (SSR) markers. A total of 81 alleles were detected with the 26 SSR markers, with an average of 3.12 alleles per locus and a PIC value varying from 0.028 to 0.450. UPGMA cluster analysis separated the entire accessions into seven major groups. Group I accommodated twenty two advanced breeding lines along with the parental variety, MR219. Eight advanced breeding lines formed the Group III while Group II, IV, V, VI and VII contained either a single improved line or variety. Group II was constituted by the improved line G33, while Group IV and V were constituted by the released varieties MR253 and MR263, respectively. On the other hand, two TGMS lines *viz.* IR73827-23-26-15-7S and IR77271–42–5–4–36S were clustered under Group VI and Group VII, respectively. Based on the genetic distance (GD) data derived from the analysis, three combinations with maximum GD *viz*., MR253 x G33 (GD= 0.61), MR253 x G02 (GD= 0.61) and MR253 x G16 (GD=0.56) are suggested to be used as parental lines in two line hybrid rice breeding system. Besides GD, the suggested lines possess promising yield and yield related traits. The released variety, MR253 is suggested to be developed as a TGMS line. These suggested parental lines are expected to produce highest hybrid vigour and will be useful for future breeding programmes.

Key words: Agronomic traits, Cluster analysis; Genetic distance; Heterosis; Microsatellite markers

INTRODUCTION

Hybrid rice technology provides an effective approach for increasing rice production to meet growing demand for rice worldwide. Since rice is a self-pollinated crop, it requires a male sterility system in order to produce commercial rice hybrids. The first commercially usable cytoplasmic male sterile (CMS) line was developed in 1972, from a sterile male plant identified from a wild rice population (Yuan, 1977). However, the CMS lines introduced in Malaysia in 1985 were found to have unstable pollen sterility and low outcrossing potential (Guok, 1994). Consequently, two-line hybrid breeding system utilizing thermo-sensitive genic male sterility (TGMS) was proposed as a viable alternative to CMS based three line breeding, due to more stable self seed production (Virmani *et al.,* 2003).

* To whom correspondence should be addressed.

When concerned with traits such as growth rate, reproductive success and yield, heterosis often produces increased phenotypic superiority of hybrids over its parents (Lippman & Zamir, 2006). Molecular marker analysis revealed that broadening genetic base of plant material can increase the magnitude of heterosis (Singh *et al.,* 2008). A positive correlation between genetic distance and heterosis was also reported in rice (Cai *et al.,* 2005). Therefore, estimation of genetic diversity among rice genotypes plays a vital role in selecting parents with wide variability for different agronomic characters. Microsatellites are widely used as markers for detecting heterosis due to their rapidity of use, rich polymorphic potential, high stability and codominant nature (Sajib *et al.,* 2012; Zhang *et al.,* 2007). The efficiency of predicting heterosis can be improved by utilizing markers tightly linked to QTLs influencing heterosis for the target trait (Charcosset & Essioux, 1994). The present study was conducted to evaluate the genetic relationship

among 31 advanced breeding lines of crosses between *O. rufipogon* and MR219 along with three Malaysian high yielding varieties and two thermosensitive male sterile (TGMS) lines based on 26 SSR markers. Several yield and yield related traits were also evaluated to observe the potential of the lines. This analysis excluded the varieties (MR253, MR263, TGMS1 and TGMS2). This evaluation would further facilitate in identifying efficient parental lines for producing two-line hybrid rice with high level of heterosis.

MATERIALS AND METHODS

Plant materials and DNA isolation

Thirty one advanced breeding lines derived from crosses between *O. rufipogon* and MR219 (Sabu *et al.,* 2006), three high yielding varieties (MR219, MR253 and MR263) and two thermosensitive male sterile (TGMS) lines (IR73827-23-26-15-7S and IR77271-42-5-4-36S) were used (Table 1). MR219, MR253 and MR263 were obtained from Malaysian Agriculture Research and Development Institute (MARDI), Malaysia whereas the TGMS lines (IR73827-23-26-15-7S and IR77271-42-5-4-36S) were obtained from International Rice Research Institute, Philippines.

Genomic DNA was isolated and purified from fresh young leaves of 36 rice samples using DNeasy Plant Mini Kit (Qiagen, Germany). The DNA concentration of each sample was checked using spectrophotometer (Eppendorf Biophotometer, Germany).

SSR marker analysis

Twenty six SSR markers linked to different agronomic traits (Table 2) were used in the study. The PCR mix (12.5 µl) contained 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.2 µM of SSR primers, 0.5 U of *Taq* DNA polymerase (iNtRON Biotechnology Inc, Korea) and 5 ng of genomic DNA. Amplification of the target DNA was performed in a thermal cycler (Eppendorf, Germany) programmed for an initial denaturation for 5 min at 94ºC followed by 35 cycles of (1 min at 94ºC, 1 min at 55ºC, 2 min at 72ºC) and a final extension for 7 min at 72ºC. The PCR products were resolved in 10% non-denaturing polyacrylamide gel at 100 W for 2 hours along with 50-bp and 100-bp DNA ladder (MBI Fermentas, Maryland, USA). The gels were either stained with ethidium bromide or silver stained (Benbouza *et al.,* 2006) and documented using Alpha Imager gel documentation system (Alpha Innotech, San Leandro, USA).

The genotypes were scored visually by comparing the molecular (band) size of the sample with that of MR219 and *O. rufipogon*. Each band was treated as an independent allele. The molecular size of amplified fragments was estimated with the aid of Gel Analyser (*Sequentix*, Klein Raden, Germany). The band was assigned as 'A', 'B', 'C', 'D' or 'E' according to different fragment sizes. The genotypes of different individuals were hypothetically scored as AA, BB, CC, DD, EE etc for homozygous and AB, AC, BC etc for heterozygous condition.

Plant materials for phenotypic evaluation

The 31 advanced breeding lines and Malaysian cultivar MR219 were evaluated for yield and yield related traits. The data values were obtained from the average value of two evaluating seasons for the specific trait. The plants were grown in the farmer's field and the intercultural management was done following farmer practices.

Data analysis

The polymorphism information content (PIC), number of alleles per locus and frequency of major allele for each SSR marker were calculated using PowerMarker ver. 3.25 software (Liu & Muse 2005). The PIC value for each SSR marker was calculated using the formula

$$\widehat{\mathrm{PIC}} = 1 - \sum_{u=1}^{k} \tilde{P}_{lu}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{P}_{lu}^2 \tilde{P}_{lv}^2$$

where P_{lu} and P_{lv} is frequency of uth and vth allele for marker l and the summation extends over k alleles (Botstein *et al.,* 1980). PIC value is a measure of relative informativeness of a marker (Botstein *et al.,* 1980).

Table 1. Details of the rice lines and varieties and the code used in the experiment

Code	Name	Code	Name
G01	R1-1-1-1	G19	R17-1-83-3
G02	R1-2-2-1	G20	R19-4-95-3
G03	R2-7-15-5	G21	R19-4-96-3
G04	R2-10-18-2	G22	R19-5-96-4
G05	R4-9-27-1	G23	R19-9-98-5
G06	R4-9-27-4	G24	R24-10-106-1
G07	R6-2-31-2	G25	R26-2-108-1
G08	R7-6-38-2	G26	R26-6-113-1
G09	R7-7-39-4	G33	R14-3-66-4
G10	R8-9-49-10	G34	R14-10-70-4
G11	R9-5-55-3	G35	R16-1-77-4
G12	R9-9-58-2	G36	R19-2-93-2
G13	R14-9-69-2	G37	R19-2-93-3
G14	R14-9-69-3	MR219	MR219
G15	R14-9-69-4	MR253	MR253
G16	R14-9-69-5	MR263	MR263
G17	R16-8-80-2	TGMS1	IR73827-23-26-15-7S
G18	R17-1-83-2	TGMS2	IR77271–42–5–4–36S

Table 2. Details of SSR markers used in the present study to estimate the genetic diversity of 36 rice genotypes

Marker	Agro-traits	Motif repeat	Product size
RM3	PPL, TPL	$(GA)_2GG(GA)_{25}$	149
RM5	GPL, DTM, CL	$(GA)_{14}$	113
RM25	SPL, GPL	$(GA)_{18}$	146
RM60	CL, PH, DTH	$(AATT)_5AATCT(AATT)$	159
RM101	SPP	$(CT)_{37}$	324
RM156	PSS, GW,DTH	$(CGG)_8$	160
RM157	SPP	$(CT)_{11}(TC)_{10}$	106
RM174	PL	$(AGG)_7(GA)_{10}$	208
RM209	DTM	$(CT)_{18}$	134
RM246	CL	$(CT)_{20}$	116
RM259	SPP, SPL	$(CT)_{17}$	162
RM260	DTM	$(CT)_{34}$	111
RM273	DTM	$(GA)_{11}$	207
RM294	CL, PH	$\{(GT)_3T_2AGGGACA\}_2$	173
RM297	CL, PH	$(GA)_{13}$	148
RM303	YLD	$[AC(AT)_{2\text{-}10}]_9(GT)_7(ATGT)_6$	200
RM309	DTM, DTH	$(GT)_{13}$	169
RM313	DTH	$(GT)_6CA(CG)_{5\text{-}6}\text{-}(GT)_8$	111
RM403	DTM	$(GA)_8$	241
RM452	PL	$(GTC)_9$	209
RM488	CL, SPL	$(GA)_{17}$	177
RM493	DTH, DTM	$(CTT)_9$	211
RM514	DTH	$(AC)_{12}$	259
RM517	SPP	$(CT)_{15}$	266
RM536	DTH	$(CT)_{16}$	243
RM540	GW, DTM	$(AG)_{16}$	172

CL=culm length, DTH = days to heading, DTM =days to maturity, GPL= grain per plant, GW= 1000-grain weight, PH= plant height, PL=panicle length, PPL =panicle number per plant, PSS=Seed set percentage, SPL= spikelet number per plant, SPP = spikelet per panicle, TPL = tiller number per plant, YLD= yield tonnes per hectare

Based on the genotype data, genetic distance (GD) of pairwise combinations of 36 rice materials were computed according to Nei *et al.* (1983), $D_A = 1 - \sum_{i=1}^{m} \sum_{f=1}^{r} \frac{\sqrt{x_{ij} y_{ij}}}{r}$, where x_{ij} and y_{ij} are allele frequency *i* at locus *j*, for population X and Y, respectively. m is the number of allele per locus and r is the number of loci studied. Cluster analysis was carried out based on GD values using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA; Sneath & Sokal, 1973) and the genetic relationship among the rice accessions were visualized as dendrograms using the NTSYS-pc package (Rohlf, 1990).

RESULTS

Allelic diversity inferred from SSR analysis

A total of 81 alleles were detected with the 26 SSR markers in the present analysis of 36 rice genotypes. The number of alleles detected ranged from 2 to 5; with an average of 3.12 alleles per locus. Marker RM493 displayed the highest polymorphism in which five alleles amplified while the lowest polymorphism was displayed by seven SSR markers with only two alleles generated. The PIC value for the 26 markers ranged from 0.028 (RM60) to 0.450 (RM246) with an average value of 0.276. The frequency of the major (most common) allele at each locus ranged from 58.3% (RM25) to 98.6% (RM60). The details for major allele frequency, number of alleles and PIC values of each marker amplified among the accessions are presented in Table 3. The relative informativeness of each marker was evaluated based on its PIC value (Botstein *et al.,* 1980).

Genetic distance (GD) and cluster analysis

Analysis of the genetic relationship based on 26 SSR markers revealed that the genetic distance among 36 individual genotypes ranged from 0.00 for two pairs of accessions viz. G18 & G20, and G10 & G1, to 0.61 (G33 and MR253) with an average value of 0.33. Cluster analysis separated the 36 individuals into seven main clusters at the genetic

Table 3. Parameter of genetic diversity estimated using PowerMarker ver 3.25 software

Marker	Major Allele Frequency	Allele No.	PIC
RM3	0.833	3.000	0.269
RM5	0.750	3.000	0.331
RM25	0.583	2.000	0.368
RM60	0.986	2.000	0.028
RM101	0.653	4.000	0.437
RM156	0.914	4.000	0.154
RM157	0.597	2.000	0.365
RM174	0.833	3.000	0.258
RM209	0.889	4.000	0.198
RM246	0.667	4.000	0.450
RM259	0.778	3.000	0.310
RM260	0.712	3.000	0.357
RM273	0.903	4.000	0.176
RM294	0.819	2.000	0.252
RM297	0.792	4.000	0.317
RM303	0.900	3.000	0.174
RM309	0.875	4.000	0.220
RM313	0.757	2.000	0.300
RM403	0.681	2.000	0.340
RM452	0.800	3.000	0.291
RM488	0.736	3.000	0.340
RM493	0.722	5.000	0.396
RM514	0.868	2.000	0.203
RM517	0.829	4.000	0.286
RM536	0.886	3.000	0.195
RM540	0.903	3.000	0.170
Mean	0.795	3.115	0.276

distance value of 0.32 as depicted in the dendogram (Fig. 1). Group I was formed by twenty two advanced breeding lines including the parental variety, MR219 while Group II comprised of solely G33. Eight advanced breeding lines were clustered together under Group III. Group IV to VII was represented by single accession each. Two varieties, MR253 and MR263 constituted Group IV and V respectively while two TGMS lines, IR73827-23-26-15-7S and IR77271–42–5–4–36S formed Group VI and Group VII respectively.

Among the 36 rice materials analysed, two pairs of advanced breeding lines viz. G18 & G20, and G10 & G1 showed similar allelic pattern with all the SSR markers used in the present study. These breeding lines thus showed 100% genetic similarity among themselves, confirming their close genetic relationships. However, G33 showed a different allelic profile from other advanced breeding lines especially for markers RM303, RM313 and RM493. Therefore, it was grouped separately from all other advanced breeding lines in the dendrogram. Two cultivars from MARDI (MR253 and MR263) and the TGMS lines (IR73827-23-26-15-7S and IR77271–42–5–4–36S) from IRRI were found to be genetically distinct from all other accessions used in the study. TGMS lines IR73827-23-26-15-7S and IR77271–42–5–4–36S were clearly distinct from the rest of the accessions at many loci (Table 4). The breeding line which showed maximum genetic distance (GD) with MR253 also showed promising value for yield and yield related traits (Table 5).

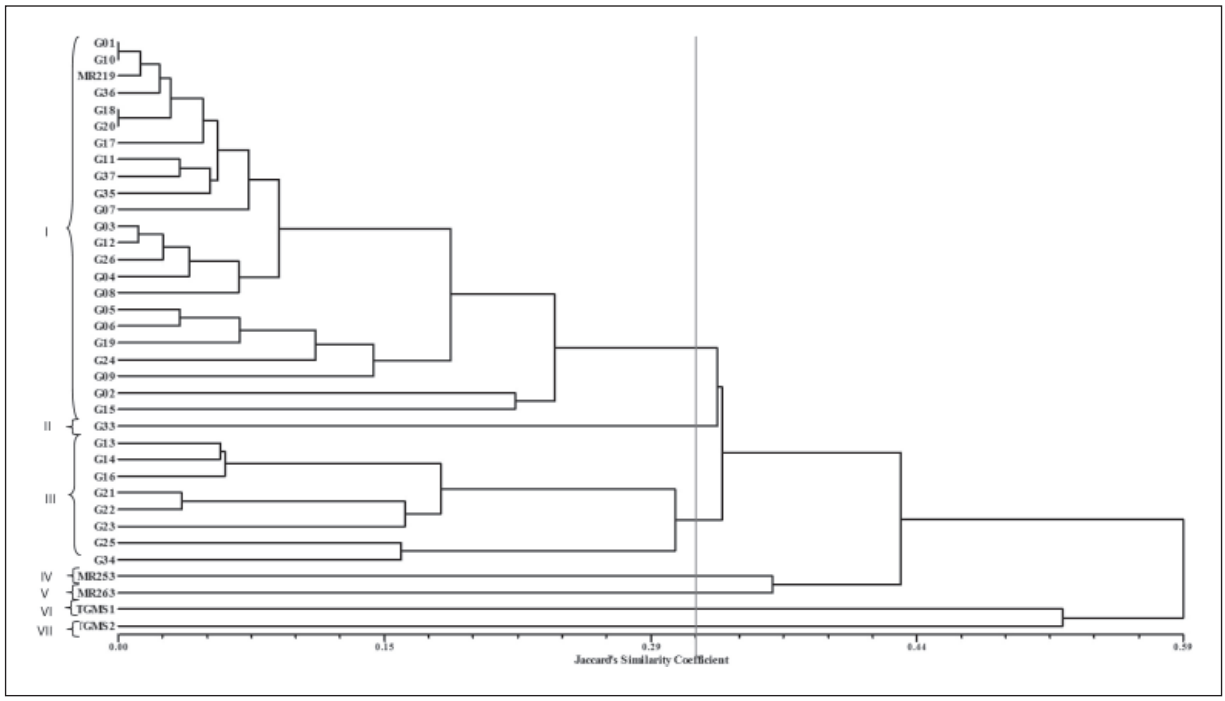

Fig. 1. Dendogram showing genetic relationship among 36 rice genotypes.

Table 4. Markers showing distinct polymorphic SSR profiles based on agronomic traits

Group	Markers
Group I	RM5, RM246, RM294, RM297, RM403, RM488
Group II	RM303, RM313, RM493,
Group III	RM5, RM246, RM297, RM403, RM488
Group IV	RM101, RM246, RM259, RM260, RM309, RM517
Group V	RM101, RM246, RM303, RM493, RM517
Group VI	RM3, RM101, RM209, RM246, RM273, RM297, RM309, RM452, RM488, RM493, RM517
GroupVII	RM3, RM5, RM156, RM174, RM209, RM246, RM273, RM297, RM309, RM493, RM517, RM536, RM540

Table 5. Yield and yield related trait value of the evaluated advanced breeding lines

Rice lines	PH (cm)	DTM (days)	PPL	FGPL	PL (cm)	HI	TGW (gm)	YPL (gm)
G01	114	122	18	1629	22.8	0.46	27.6	44.33
G02	126	120	20	1802	23.1	0.53	29.1	51.33
G03	120	119	16	1745	23.0	0.55	28.4	49.50
G04	117	120	15	1839	23.6	0.55	28.1	51.58
G05	122	121	16	1794	24.1	0.50	26.6	47.23
G06	124	122	18	2050	24.5	0.50	26.3	53.87
G07	126	120	19	2180	23.8	0.51	26.5	54.77
G08	121	121	18	1747	23.4	0.55	30.9	53.85
G09	128	121	15	1838	22.8	0.55	26.5	48.57
G10	117	122	16	1959	23.9	0.51	25.4	49.80
G11	120	123	16	2001	23.8	0.51	25.3	50.95
G12	120	120	15	1759	23.4	0.55	29.8	52.41
G13	118	119	16	1799	23.4	0.53	26.9	49.00
G14	123	120	16	1776	23.9	0.51	27.1	48.22
G15	118	118	17	1587	22.7	0.53	28.6	45.13
G16	125	120	18	1770	23.9	0.51	26.8	45.35
G17	118	121	15	1766	23.5	0.50	26.9	47.53
G18	115	121	18	1815	22.9	0.48	27.6	50.53
G19	118	123	17	1795	23.0	0.55	28.3	51.28
G20	118	121	15	1715	22.9	0.55	26.9	46.45
G21	125	121	17	1881	22.8	0.50	25.4	47.85
G22	124	120	17	1856	22.4	0.50	24.7	45.77
G23	120	120	17	1655	22.2	0.51	27.1	45.28
G24	126	121	17	1531	22.8	0.48	27.3	41.90
G25	118	122	16	1752	23.8	0.50	27.5	47.57
G26	116	118	15	1785	22.8	0.55	27.4	49.03
G33	105	125	20	1660	26.2	0.50	22.3	42.11
G34	104	124	15	1432	25.9	0.50	20.9	37.82
G35	102	124	17	1425	24.1	0.46	22.7	35.10
G36	105	124	16	1378	24.2	0.45	23.7	33.22
G37	106	124	18	1537	24.5	0.45	23.0	37.02
MR219	119	123	14	1843	24.0	0.50	26.1	46.40

PH = Plant height, DTM = days to maturity, FGPL = Filled grain per plant, GW = 1000-grain weight, PL = panicle length, PPL = panicle number per plant, HI = Harvest index, YPL = yield per plant

DISCUSSION

UPGMA cluster analysis of 36 rice lines clearly distinguished majority of the advanced breeding lines, from MARDI's new varieties (MR253 and MR263) and IRRI's TGMS lines (IR73827-23-26-15-7S and IR77271–42–5–4–36S). SSR genotyping separated the entire accessions into seven major groups (I, II, III, IV, V, VI and VII) at the genetic distance value of 0.32 (Fig. 1). In general, the grouping of accessions was in accordance to agronomic and phenotypic characters.

In spite of deriving from the same crosses viz. MR219 and *O. rufipogon*, the clustering pattern of advanced breeding lines into three separate groups - I, II and III based on the genotype data was clearly

reflected in terms of agronomic characteristics, thousand-grain weight. The members belonging Group I was mainly characterized by the largest grain size whereas eight advanced breeding lines belonging to Group III yielded intermediate grain size. On the other hand, genotype G33 under Group II showed smaller grain size (Table 5).

Inclusion of the parental line MR219 within Group I indicated close genetic relationship between the twenty two advanced breeding lines with the parent MR219. In accordance, since the microsatellites used in the study were related to agronomic traits, the advanced breeding lines under Group I were also expected to show similar or better performance to MR219 in certain agronomic traits such as grain weight, yield per plant and panicle length. Besides showing high performance in terms of thousand-grain weight, Group I contained early maturing lines with higher yield than MR219 (Table 5). It is evident that interaction of genes and subsequent recombination events between *O. rufipogon* and MR219 might have resulted in an increased yield as suggested by Bhuiyan *et al.* (2011). Similar results were also reported by Septiningisih *et al.* (2003) and McCouch *et al.* (2007). However, some variations in the actual performance of agronomic traits might be due to genotype x environment interactions (Sabu *et al.,* 2009).

In spite of its common origin, the separate grouping of G33 (Group II) from other rice lines is also quite interesting. The demarcation of genotype G33 from MR219 parent was attributed to several microsatellite loci *viz*. RM25, RM101, RM260, RM303, RM313 and RM452. This variation was also clearly displayed in agronomic characters with respect to red pericarp percentage (99%), where it was found to be the highest among all red pericarp rice lines including the parental line, MR219. G33 was also characterized by its shorter plant height, late maturing line and longer panicle, when compared with other advanced breeding lines (Table 5). These observations support the separate clustering of G33 from other accessions as obtained through the use of the markers.

On the other hand, eight advanced breeding lines (Group III) were found significantly different from MR219 at several microsatellite loci viz. RM5, RM101, RM157, RM246, RM294, RM297, RM403 and RM488. The corresponding difference was also reflected in some of morphological and agronomic characteristics such as plant height, number of panicle per plant and yield (t/ha) (Bhuiyan, 2010).

The identical allelic profiles revealed by two pairs of advanced breeding lines *viz*. G1 x G10, and G18 x G20 with all SSR markers indicate homogenous nature of genetic material of these lines. Thus, these lines are expected to show similar agronomic traits. For instance, the trait data of G18 and G20 revealed almost similar performance for characters such as days to maturity (121 days) and panicle length (22.9cm), respectively. Both G1 and G10 also showed similarities in terms of days of maturity which is 122 days (Table 5).

Another interesting feature revealed in the study was the separate grouping of Malaysian rice varieties, MR253 and MR263. Both these varieties are completely different from all of the other accessions and displayed only 64% similarity among them. This is in agreement with the report by Mazid *et al.* (2013), where MR253 and MR263 were separated into two different groups based on thirteen morphological traits studied such as number of filled grain panicle, yield per hill and thousand-grain weight per hill. MR253 was also reported as an early maturing variety possessing resistance towards leaf blast disease and produced higher yield per hill and thousand-grain weight per hill when compared to MR263. However, the genetic relationship between them could not be confirmed due to the lack of substantial pedigree information.

Since TGMS lines IR73827-23-26-15-7S and IR77271–42–5–4–36S showed distinct allelic pattern from the rest of the genotypes at many loci, it is expected that the two TGMS lines will also perform differently from each other and other accessions as well in terms of some morphological and agronomic traits. For instance, based on pollen sterility and spikelet sterility, the TGMS lines IR73827-23-26-15-7S and IR77271–42–5–4–36S were clearly differentiated from the rest of the accessions. At a particular sterility-inducing temperature, the TGMS lines may show complete pollen sterility and might be able to revert to fertility, when it is exposed to fertility-inducing temperature since the expression of fertility or sterility is mainly controlled by the environmental temperature (Zhang *et al.,* 2009).

Among the rice lines used in the study, three combinations of potential parental lines for producing hybrid rice with farthest genetic distances include MR253 x G33 (GD= 0.6117), MR253 x G02 (GD= 0.6077) and MR253 x G16 (GD=0.5600). The breeding lines included in the combinations showed wide variation in several morphological and agronomic traits such as plant height, days to maturity, thousand-grain weight, filled grain per plant, harvest index and yield per plant (Table 5; Mazid *et al.,* 2013). These potential parental combinations are expected to show high magnitude of hybrid vigour and increased yield, when compared to high yielding varieties. The variety, MR253 is also suggested to be developed as a TGMS line.

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