Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

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Asian Journal of Plant Sciences 7 (2): 140-148, 2008
ISSN 1682-3974

Variation of Microsatellite Markers in a Collection of Lao's Black Glutinous Rice (*Oryza sativa* L.)

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Abstract: The genetic diversity of 74 genotypes, including Black Glutinous Rice (BGR) from Lao's germplasm was assessed using 24 microsatellite markers. A total of 75 alleles were detected at the 24 microsatellite markers. The number of alleles per marker varied from 2 to 7 with an average of 3.1 alleles per locus. The Gene Diversity (GD) and Polymorphism Information Content (PIC) ranged from 0.18 to 0.79 and 0.17-0.76, respectively and the Allele Frequency (AF) ranged from 0.36 to 0.90. The markers were able to classify rice genotypes into four groups; *indica* rices were put in the three groups while the other group consisted of tropical *japonica* rice. The first *indica* group (G1) included 24 genotypes of BGR and five genotypes of white rice. Most of genotypes in this group have thick culms, broad leaf blades, large and bold grain shapes and some of them have purple coloration on all vegetative parts. In other varieties with black pericarp, all other plant parts are green. *Indica* group 3 (G3) included 25 genotypes of BGR. The special characters of this subgroup were small and slender culms, narrow short leaves, purple leaf margins, purple leaf tips and purple stripes on leaf blades and sheathes. However, the markers used could not differentiate between LG 8215 and LG 7937. *Indica* group four (G4) consisted of eight genotypes of white rice and the four check varieties. Group (G2) consisted of five white rices, four BGRs and three check varieties.

Key words: Laos, germplasm diversity, SSR, *indica*, tropical *japonica*

INTRODUCTION

Rice belongs to the genus *Oryza* in the grass family (Gramineae). There are 22 species in the genus, of which only two are cultivated: *O. sativa*, which was domesticated in the humid tropics of South and Southeast to East Asia and *O. glaberrima*, which was domesticated in the Niger basin in Africa (Khush, 1997). *O. sativa* was domesticated in south Asia at least 10,000 years ago (Zhang and Jiarong, 1998) and *O. glabberima*, was domesticated in West Africa between about 1500 and 800 BC (Murrey, 2004).

South and Southeast Asia is generally accepted as the center of origin and domestication of glutinous rice and glutinous rice varieties are grown in most countries in these areas that have long history of rice cultivation. Laos has the highest per capita production and consumption of glutinous rice in the world (Schiller *et al*., 2006). Lao PDR proved to have one of the richest biodiversities in rice and it appears to be the world's biodiversity centre for glutinous (sticky) rice varieties (Anonymous, 2007). In Lao PDR, traditional varieties of black glutinous rice are named by farmers for a particular distinctive character or region and a single name may encompass a genetically diverse set of varieties. For example, rice varieties with coloured pericarps other than red are usually called Khao Dam or Khao Kam (Dark Rice or Black Rice) (Appa *et al*., 2002a).

The black rices of Laos are less in production and consumption, but they are not least in their importance. They have long been cultivated and are widely grown in small plots throughout the country for making special recipes in special occasions such as bamboo rice, rice cakes and special alcohol drinks. BGR varieties can command a premium price in both the international and domestic markets. Their superior characteristics, such as high eating quality and grain characteristics and good general adaptability to biotic and abiotic factors, make them popular among Lao farmers.

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The Lao Ministry of Agriculture and Forestry (MAF) and the International Rice Research Institute (IRRI) had jointly collected 13, 992 samples of cultivated rice germplasm in Laos during 1995 to 2003 (Appa *et al*., 2002a, b) and 431 accessions (3.07% of the total collections) were classified as having coloured pericarps based on the information provided by the farmers at the time of collection of the germplasm and on the characterization data from the Agriculture Research Center (ARC), (ARC, 2003, 2004; Appa *et al*., 2003). This germplasm is currently conserved in the Lao's national genebank and in the International Rice Germplasm Collection (IRGC).

In an attempt to best utilize this indigenous rice germplasm, several activities for short-term application have been initiated. These activities include primary characterization, evaluation in replicated yield trails (Inthapanya *et al*., 1995). For the longer term utilization of the germplasm, it is necessary to quantify genetic diversity among Lao BGR varieties and to classify the genetic differentiation among them at molecular level. Most studies have been conducted so far with non-glutinous rice, fragrant rice and white glutinous rice using several types of molecular markers. The lowest genetic diversity was observed among the traditional basmati varieties, whereas the evolved basmati varieties showed the highest genetic diversity by Simple Sequence Repeats (SSR) and Inter Simple Sequence Repeats (ISSR) assays (Nagaraju *et al*., 2002). Bao *et al*. (2006) reported that high levels of AFLP (78.3%) and ISSR (92.2%) polymorphism were found in 56 waxy (glutinous) rice accessions. In general, gene diversities in rice populations (landraces, old varieties, newly developed varieties, wild species and parental lines of hybrids) as measured by different types of molecular markers ranged from 32.0 to 70.0 depending on methods used and rice populations under study (Giarrocco *et al*., 2007; Pessoa *et al*., 2007; Xu *et al*., 2004; Yu *et al*., 2003; Coburn *et al*., 2002; Ni *et al*., 2002) except for very low genetic diversity (PIC = 0.001) of landrace accessions in high altitude regions of Nepal (Bajracharya *et al*., 2006).

Most studies identified *indica-japonica* pattern of genetic diversity of Asian rice. In a study on a study of a wide range of rice genotypes using nuclear SSRs and two chloroplast loci, Garris *et al*. (2005) found five distinct groups corresponding to *indica*, *aus*, *aromatic*, temperate *japonica* and tropical *japonica* rices. They also found that *indica* was closely related with *aus*, whereas tropical *japonica*, temperate *japonica* and aromatic rices were closely related. In a study in Indonesia conducted on traditional and improved varieties, Thomson *et al*. (2007) found that *indica* rice (68%) predominated over tropical *japonica* rice (32%) in total number of accessions studied but gene diversities were rather similar (0.53 and 0.56 for *indica* and tropical *japonica*, respectively). Using combined AFLP and SSR markers, Bao *et al*. (2006) could clearly differentiate *indica* and *japonica* groups of waxy rice accessions.

Unfortunately, this very important information for black glutinous rice is not available in the literature. This is because most rice breeding programs have been focused on non-glutinous rice and breeding of glutinous rice especially for black glutinous rice is far behind. The objective of this study was to use SSR markers to quantify genetic diversity among Lao BGR varieties and classify the genetic differentiation among them. The information will be useful for future breeding program, conservation and effective management of black rice genetic resources.

MATERIALS AND METHODS

Plant materials: The research was undertaken in 2006 at the IRGC at the International Rice Research Institute (IRRI), Philippines. Rice varieties used for this study consisted of 56 varieties of *Oryza sativa* provided by the Lao's national genebank (Table 1) and 18 varieties of standard white rice supplied by IRGC. Fifty three varieties out of the total of 74 varieties were tested and classified into black rice. The remaining consisted of tropical *japonica*, *indica* rice and white glutinous and non-glutinous rice.

DNA extraction: Total genomic DNA was extracted from young leaves using a modification of the method described by Fulton *et al*. (1995). Approximately 5 mg of young leaf tissue was frozen in liquid nitrogen, followed by grinding using a plastic bit attached to an electric drill before addition of extraction buffer, which was different from that of Fulton *et al*. (1995) who used mortar and pestle. Other steps in extraction followed Fulton *et al*. (1995). After extraction, pellets were dissolved in 150 μL TE (10 mM Tris, 1 mM EDTA, pH 8). DNAs were quantified by gel densitometry on agarose gels using Lambda DNA as a standard followed by normalization to concentration of 5 ng μL^{-1} prior to use in PCR.

PCR amplification: Polymerase chain reactions using microsatellite primers were carried out in a final volume of 10 μL. The PCR reaction mixture contained 1 μL 10xTBE buffer (containing 100 mM Tris-HCl pH 8.5, 50 mM KCl), 1 μL 15 mM $MgCl_2$, 1 μL of 5 mM dNTP mixture, 1 μL each of 10 mM forward and reverse primers, 1 unit (0.5 μL) of Taq DNA polymerase, 10 ng of DNA template and ddH_2O to reach the final reaction volume of 10 μL. Reactions were overlaid with mineral oil.

Table 1: Some agronomic characters of 53 accessions of lowland black glutinous rice and three varieties of Lao white rice (6732 = Kai Noi Leuang, 1655 = Hom Nang Nouan and TDK5)

Accession No.	Days to flowering	Panicle length (cm)	Culm length (cm)	Culm No.	100 grain weight (g)	Yield (kg ha^{-1})
175	134	28.3	135.2	7.3	2.5	2860
587	133	28.9	136.5	8.6	2.7	3167
1292	129	27.5	142.7	6.2	3.0	2503
1267	127	28.9	143.0	6.1	2.7	2441
2156	133	26.7	124.5	7.2	2.6	3253
1284	129	28.0	136.4	6.6	2.8	2533
1357	130	24.0	123.7	6.1	2.6	2455
1389	120	25.4	132.2	7.9	3.1	2726
2198	139	28.5	135.8	5.8	2.6	3179
2236	115	25.9	126.8	6.8	2.4	2236
2419	133	27.0	120.3	6.7	2.6	3290
2454	115	26.0	135.7	6.7	2.9	2292
2816	104	27.6	133.2	7.3	3.1	1548
2751	110	26.1	115.1	6.4	2.6	1880
2940	115	27.8	114.3	6.9	2.4	2412
13552	140	26.1	123.1	5.5	2.6	1426
3302	131	25.7	119.9	6.2	2.6	2757
3762	101	26.4	129.7	8.5	2.8	1854
4263	125	28.2	144.3	7.0	2.9	2725
4998	125	27.2	137.4	6.3	2.9	2601
5048	105	27.9	125.7	7.1	2.8	1605
5142	132	26.5	109.4	7.1	2.7	3106
5405	133	26.0	125.5	8.2	2.7	2866
5448	130	27.8	124.6	4.9	2.7	2302
5649	127	28.4	130.1	5.7	2.9	2032
5925	110	25.7	117.9	6.8	2.4	1632
6103	118	26.1	122.0	7.8	2.6	2259
6730	115	26.0	134.3	7.9	2.9	2226
6740	110	26.3	130.9	7.7	2.4	1310
6822	114	28.9	134.7	6.6	2.8	1574
6828	110	28.5	134.8	6.3	2.9	1551
7206	107	27.3	133.9	6.9	2.9	1628
7694	128	26.5	130.8	6.3	2.5	3067
7697	129	27.9	130.0	6.2	2.7	2705
7712	128	27.4	123.9	6.2	2.7	2984
7897	106	28.0	125.8	8.6	2.6	2129
7918	132	28.3	133.8	7.3	2.9	2840
7926	127	26.6	122.8	5.7	2.8	2786
7937	127	29.0	126.2	6.9	2.6	2949
7942	130	28.3	132.9	6.2	2.6	2646
8002	132	25.7	132.8	8.3	2.9	3289
8140	132	26.3	131.2	6.3	2.9	2720
8211	129	24.7	123.3	6.2	2.4	2594
8215	129	26.9	130.1	6.5	2.7	2262
8244	126	26.0	126.4	6.1	2.8	2876
8547	129	28.3	128.3	6.3	2.5	2688
8862	132	26.5	114.4	6.8	2.9	2728
9824	125	28.2	128.3	6.3	2.7	2667
11193	136	29.0	139.2	4.5	2.9	1987
12829	133	28.5	133.1	7.3	2.8	2650
12823	123	28.3	131.8	5.5	2.8	2503
8246	130	27.3	137.3	5.9	2.7	2893
13259	129	28.1	128.3	6.5	2.7	2542
6732	107	26.9	137.3	5.9	2.7	1329
1655	132	29.1	112.1	6.7	2.7	3604
TDK5	102	28.5	144.2	6.3	2.8	2298

To optimize the PCR amplification conditions, experiment were carried out with varying quantity of $MgCl_2$ (15 mM) and Taq DNA polymerase, two different quantity of $MgCl_2$ and Tag polymerise were used (1-1.5 and 0.5-1 μL) respectively.

The 35 SSR markers were screened in this study. Eleven SSR markers were not polymorphic and discarded. Twenty four polymorphic markers were used to amplify DNA products (Table 2). These markers were reported previously (Temnykh *et al*., 2000). Amplifications were carried out using MJR DNA Engine Dyad® thermal cyclers. The PCR programs consisted of the following steps: initial denaturation for 5 min at 94°C, followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at

Table 2: Total number of alleles, allele length in bp, gene frequency and gene diversity of 24 SSR markers assayed in 74 rice genotypes

Marker	Major AF	Genotype No.	SS	No. of obs.	Allele No.	Gene diversity	PIC	AL in (bp)	AT (°C)
RM1	0.47	5	74	74	4	0.68	0.63	100-400	55
RM5	0.58	3	74	74	3	0.50	0.39	100-220	55
RM11	0.59	4	74	74	4	0.54	0.46	180-300	55
RM19	0.50	2	74	73	3	0.59	0.50	120-350	55
RM25	0.55	3	74	74	3	0.56	0.48	100-180	57
RM55	0.54	2	74	71	2	0.50	0.37	100-180	57
RM105	0.77	2	74	74	2	0.35	0.29	100-160	61
RM152	0.64	3	74	74	3	0.50	0.43	100-200	55
RM161	0.89	3	74	74	2	0.20	0.18	100-150	61
RM162	0.49	3	74	74	3	0.63	0.56	150-400	63
RM171	0.56	5	74	74	4	0.59	0.53	110-320	55
RM215	0.50	3	74	74	3	0.59	0.51	120-210	53
RM259	0.36	13	74	74	7	0.79	0.76	100-550	55
RM271	0.37	5	74	71	5	0.74	0.69	100-350	55
RM287	0.46	3	74	74	3	0.64	0.57	150-250	55
RM307	0.54	3	74	70	3	0.53	0.43	100-320	57
RM316	0.88	2	74	74	2	0.21	0.19	210-350	55
RM408	0.55	3	74	74	3	0.57	0.50	110-200	55
RM431	0.44	4	74	74	3	0.61	0.52	200-500	53
RM447	0.61	3	74	72	3	0.55	0.49	100-180	55
RM489	0.90	3	74	74	2	0.18	0.17	130-250	55
RM510	0.86	4	74	74	3	0.25	0.22	120-350	57
RM514	0.68	4	74	74	3	0.45	0.36	300-500	55
RM552	0.77	2	74	74	2	0.35	0.29	100-190	55
Mean	0.60	3.6	74	73.4	3.1	0.50	0.44		
Max	0.90	13.0	74	74	7	0.79	0.76		63
Min	0.36	2	74	70	2	0.18	0.17		53

Remark: AF = Allele Frequency, AL = Allele Length in bp, SS = Sample Size, AT = Annealing Temperature and PIC = Polymorphic Information Content

55°C, 2 min extension at 72°C, followed by a final extension of 5 min at 72°C and holding at 15°C until recovery. For some specific microsatellites, a different annealing temperature of 53, 57, 61 or 63°C was used as indicated in Table 2.

Amplification products were separated by non-denaturing PAGE on mini vertical units using either 6.5 or 8% polyacrylamide gels with 1x TBE buffer at 100 volts for 1.5 h. After electrophoresis, gels were stained with ethidium bromide and visualized by UV illumination. Gel images were photographed using Bio-Rad GelDoc system. The molecular weights of PCR products were estimated relative to a 1 kb ladder that served as the size standard.

Data analysis: Summary statistics (PIC, GD and AF) on the marker data were calculated using PowerMarker 3.25 (Liu and Muse, 2005).

The allelic state encoded SSR data were used to calculate a matrix of genetic distances between all pairs of varieties using simple matching coefficient under DARwin® 4.0 Release (2007) (Perrier *et al*., 2003). Cluster analysis was accomplished on this matrix by the unweighted neighbor joining option.

RESULTS

Twenty-four SSR markers were used for amplifying DNA segments from genomic DNA of 74 genotypes. A total of 75 alleles were scored from the 24 SSR markers. The number of alleles per primer ranged from 2 to 7. On average, 3.1 alleles per locus were observed. The Gene Diversity (GD) ranged from 0.18-0.79. The Polymorphism Information Content (PIC) ranged from 0.17 to 0.76 and the Allele Frequencies (AF) ranged from 0.36 to 0.90 (Table 2). RM259 SSR was the highest polymorphic marker with 7 alleles. The size of the amplified segment ranged from 100-150 bp for RM161 to 100-550 bp for RM259. Number of genotypes from 24 SSR markers ranged from 2-13. The PCR products amplified by RM259, RM307 and RM162 are shown in Fig. 1. The number of alleles detected by RM171, RM271 and RM259 was slightly higher than the average with 4-7 alleles.

The microsatellite markers were able to distinguish between different rice genotypes. The high degree of polymorphism of microsatellite markers allows rapid and efficient identification of rice genotypes. The microsatellite markers classified the rice genotypes into four groups; *indica* accessions were put in the three groups while the other group consisted of tropical *japonica* rice. Genotypes in *indica* group one (G1) included 24 genotypes of BGR and five genotypes of white rice. Most of genotypes in this group have thick culms, broad leaf blades, large and bold grain shapes and some of them have purple coloration on all plant parts. In other varieties with black pericarp, other plant parts including glumes and leaves are green color. *Indica* group 3 (G3) included 25 genotypes of BGR. The special characters of this subgroup were small and slender culms,

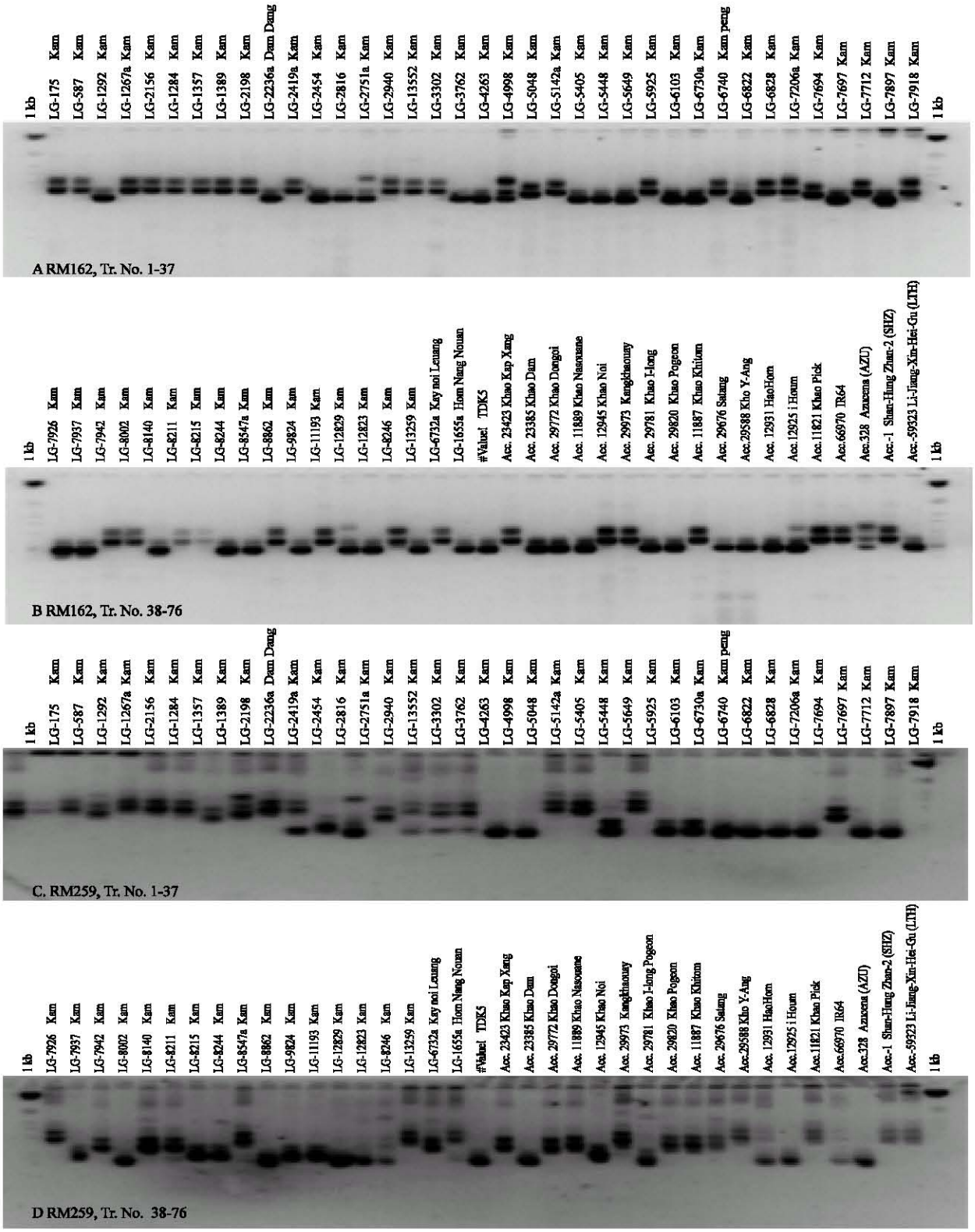

Fig. 1: SSR banding patterns of 53 black glutinous rice and 21 white rice generated from RM162 (A and B) and RM259 (C and D)

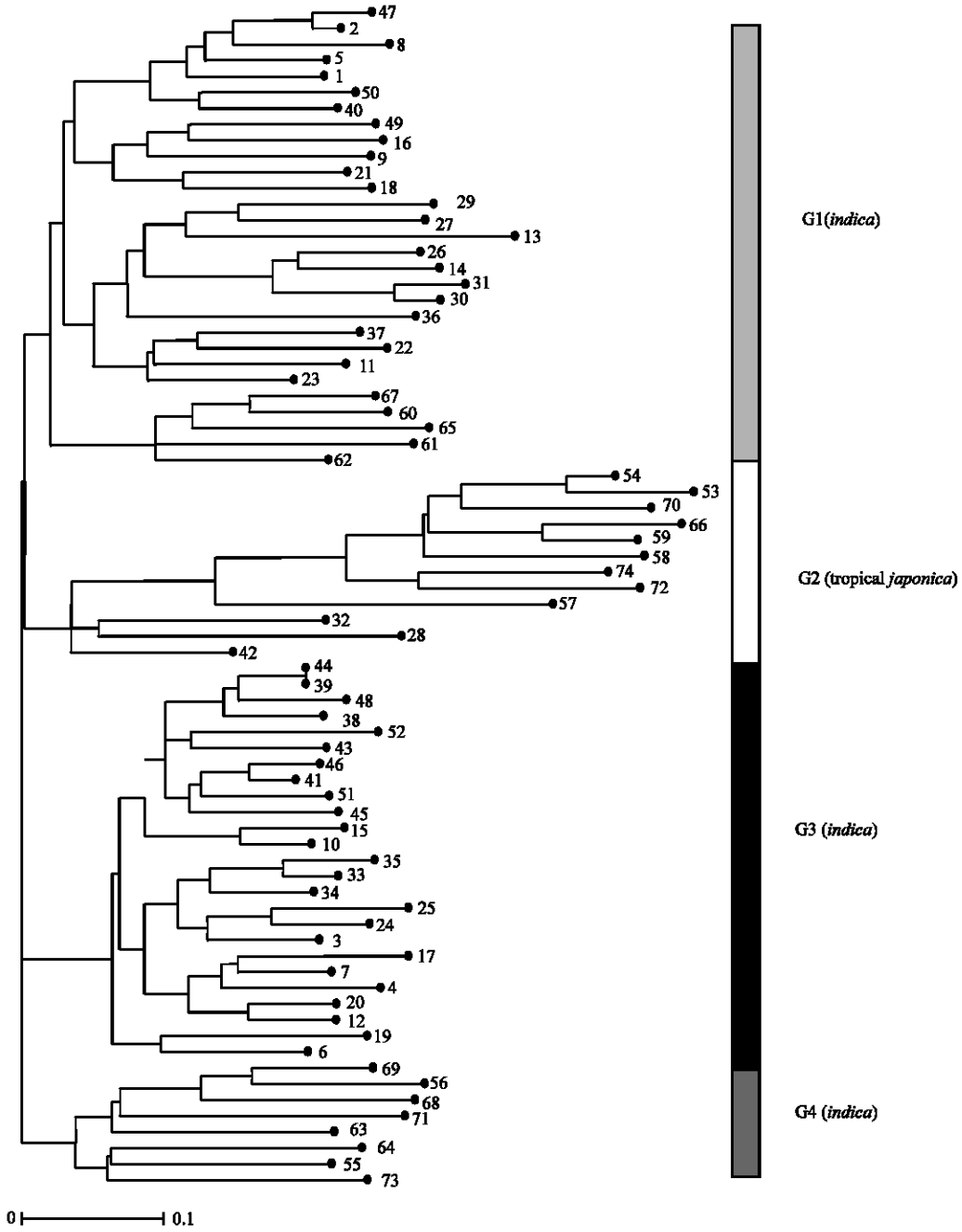

Fig. 2: Dendrogram of 74 rice genotypes, constructed using UPGMA based on simple matching coefficient using allelic states

narrow short leaves, purple leaf margins, purple leaf tips and purple stripes on leaf blades and sheaths. However two genotypes were not distinguished from each other (LG 8215 and LG 7937), these might be of the same genotype. *Indica* group four (G4) consisted of eight genotypes of white rice, which were green color with tall plant parts and included the four check varieties (Hom Nang Nouan, TDK5, IR64 and Shan-Huang Zhan-2). Tropical *japonica* group (G2) consisted of 5 genotypes of Lao white rice varieties and 4 genotypes of BGR and three check varieties (Khao Kai Noi Leuang, Azucena and Li-Jiang-Xin-Hei-Gu). Hence, the four BGR genotypes (LG6730, LG7260, LG8140 and LG13259) in this group should be tropical *japonica* types (Fig. 2).

DISCUSSION

Glutinous rice is a staple food for Lao people and black glutinous rice is also important as food for special occasions and special recipes. Black glutinous rice also serves as a valuable genetic resource for genes with economic importance such as resistance to abiotic and biotic stress, table quality and starch quality for a variety of value-added products. As a part of the center of origin and domestication of glutinous rice, high genetic diversity of this type of rice would be expected in the samples of total accessions collected in different regions of the country. The main objective of this research was to understand genetic variability of the indigenous black rice germplasm. The other objectives were to select parental genotypes for potential use in future breeding program.

Out of 35, 24 SSR primers revealed rather moderate genetic diversity of black glutinous rice as indicated by relatively low alleles per locus (3.1), GD (0.50) and PIC (0.44), when compared with the results of those available in the literature. By using 26 SSR markers, Giarrocco *et al.* (2007) found high alleles per locus (8.4) low pair-wise genetic similarity (0.32) and high PIC (0.69) in 69 rice cultivars with historical importance in Argentina. By using 47 RAPD markers, Singh *et al.* (2003) obtained a total number of 275 alleles (75 allels in present study) in 21 rainfed lowland rice genotypes consisting of historic and modern cultivars. In general, genetic diversities reported in previous studies (Xu *et al.*, 2004; Bao *et al.*, 2006; Pessoa *et al.*, 2007; Thomson *et al.*, 2007) were higher than this study but the results cannot be compared directly because neither of the previous research was conducted with black glutinous rice. However, Bajracharya *et al.* (2006) found low diversity in landrace population in Nepal with diversity index of 0.23 and these genotypes might derive from single origin. The lower genetic diversity might be caused by the following reasons. First, the samples represented only lowland black glutinous rice excluding upland black glutinous rice. Second, the samples are not well represented the total genetic variability because of low accessibility to localized areas and, if this is the case, resampling in remote sites is recommended to collect additional samples. Third, there are differences in rice populations and methods used in many studies.

Out of 35, 11 SSR primers failed to differentiate black glutinous rice samples indicated that genetic variability was not high and a large number of randomly distributed SSR primers are required to identify the genotypes. Ni *et al.* (2002) suggested that 30 SSR primers were needed to stabilize the dendrogram of 38 rice accessions. The other possible reasons for low genetic diversity in black glutinous rice are that preferential selection of farmers and genetic erosion by introduction of modern varieties might also be the causes. This highlights the importance of genetic conservation of black glutinous rice germplasm for future use. From the preliminary study on agronomic characters (Table 1), the accessions of BGR had high variations in many characters such as yield, days to flowering, panicle length and culm length. Improvement of economically important characters of this germplasm can be achieved but might be in limited extent because of low genetic diversity. Introduction of genetic materials from diverse sources is required.

Typical *indica-japonica* grouping pattern was clearly identified by the SSR dendrogram. Three groups formed an *indica* main group, whereas only one group represented *japonica* type. Higher diversity was found in *idica* rice than in tropical *japonica* rice. The results corresponded to the widely accepted grouping pattern of Asian rice (Giarrocco *et al.*, 2007; Yu *et al.*, 2003; Ni *et al.*, 2002; Glaszmann, 1987) in which six groups constitute *O. sativa* species (Glaszmann, 1987). The results were in agreement with the study of Prathepha and Baimai (2004) who found that almost all of glutinous upland rice genotypes grown by various ethnic groups in northern Thailand were characterized as *japonica* type, whereas most of rainfed glutinous lowland varieties from other regions of Thailand were *indica*. A study with Thai and Lao black glutinous rice found that based on two growing conditions, rainfed lowland and rainfed upland, chloroplast DNA type was distinct from each other (Prathepha, 2007).

To better utilize black glutinous rice in the country and achieve breeding goal, genetic diversity of upland glutinous rice should be studied and compared with this study. Black glutinous rice in other countries should be studied and introduced to broaden germplasm base of black glutinous rice.

ACKNOWLEDGMENTS

We thank Mr. Phumi INTHAPANYA Director of Agricultural Research Center of Lao PDR, for seed samples of Lao accessions. Financial support from IRRI's Training Center is gratefully acknowledged.

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