Pertanika J. Trop. Agric. Sci. 41 (1): 177 - 190 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Genetic Diversity and Relationship of Sabah Traditional Rice Varieties as Revealed by RAPD Markers

Eric Tzyy Jiann Chong, Lucky Poh Wah Goh, Jovita Jun Wong, Zaleha Abdul Aziz, Noumie @ Loumie Surugau, Mariam Abd. Latip and Ping-Chin Lee*

Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia

ABSTRACT

Sabah, also known as North Borneo, is one of the states in Malaysia. It is home to many local varieties of rice, but the self-sufficiency quotient for rice production is only about 30%. Knowledge of the genetic diversity of crops has been utilised to increase crop yields including rice in different countries, but the information regarding the genetic diversity of Sabah traditional rice varieties is very limited. Therefore, we report a comprehensive genetic diversity and relationship study of 22 Sabah traditional rice varieties in three main divisions of Sabah including the West Coast Division (WCD), Sandakan Division (SD), and Interior Division (ID) using 11 random amplified polymorphic DNA (RAPD) markers. Our results showed that more than half of the collected rice seeds were medium in size and shape, with moderately high head rice recovery and low moisture content. In addition, about half of them were categorised with high to very high amylose content. Genetic analysis revealed a total of 75 bands were produced using all RAPD markers with 100% polymorphism, and a high degree of genetic variation among all Sabah traditional rice varieties was obtained. The genetic differentiation of Sabah's traditional rice varieties was more likely to occur within divisions rather than among divisions. Furthermore, Sabah traditional rice varieties in ID showed the greatest genetic diversity and polymorphic loci, and were closely related to rice varieties in SD but genetically dissimilar to those in WCD as revealed in both phylogenetic tree and principal component analysis. In conclusion, this study provides breeders with reliable information regarding diversity

ARTICLE INFO

Article history:
Received: 29 December 2016
Accepted: 05 October 2017

E-mail addresses:
eric_ctj@live.com (Eric Tzyy Jiann Chong),
luckygoh@hotmail.com (Lucky Poh Wah Goh),
jwongjun@yahoo.com (Jovita Jun Wong),
zalehaaz@ums.edu.my (Zaleha Abdul Aziz),
lnoumie@ums.edu.my (Noumie @ Loumie Surugau),
almariam@ums.edu.my (Mariam Abd. Latip),
leepc@ums.edu.my (Ping-Chin Lee)
* Corresponding author

ISSN: 1511-3701

of Sabah's traditional rice varieties; the data could also be beneficial for local rice yield enhancement.

Keywords: Genetic diversity, Sabah's traditional rice, RAPD, phylogenetic tree, principal component analysis

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's populations (Malik et al., 2008). About 163.1 million hectares of rice are cultivated, and the worldwide annual rice production was around 748 million tons in 2016 (FAO, 2017). In Malaysia, the production of rice has only been able to meet approximately 65% of domestic needs, and the remaining 35% are imported from other countries (Vengedasalam et al., 2011). Sabah, also known as North Borneo, is one of the states in Malaysia. It is home to many local rice varieties but the self-sufficiency level is only about 30% (Mohamad Shokur et al., 2015). Hence, the National Food Security Policy launched in 2008 has identified Sabah as one of the regions in which local rice plantations and production need to be increased in order to sustain domestic demand.

Genetic diversity of crops is mainly influenced by several factors such as genetic drift, mating system, evolutionary history and life history (Loveless & Hamrick, 1984). Knowledge of genetic diversity of crops such as rice, including their wild relatives and traditional varieties, is essential for crop management, crop improvement by selection, usage of crop germplasm, genetic mapping and detection of genome structures to ensure crops with superior characteristics are planted (Sasaki, 2005; Sabu et al., 2006; Varshney et al., 2008; Pooja & Katoch, 2014). Besides, rice is an ideal crop for genetic diversity assessment due to the significant level of genetic polymorphisms present in the genome (Wang et al., 1995; Latif et al., 2011).

Recently, one study reported that rice varieties from Sabah and Sarawak (East Malaysia) were clustered together when compared to rice varieties from Peninsular Malaysia (Razak et al., 2016). However, the genetic diversity of rice in different divisions of Sabah was not investigated. Therefore, this study emphasises the genetic diversity and relationship of 22 Sabah's traditional rice varieties from three main divisions of Sabah including the West Coast Division (WCD), Sandakan Division (SD) and the Interior Division (ID) using 11 random amplified polymorphic DNA (RAPD) markers.

MATERIALS AND METHOD

Plant Materials and DNA Isolation

A total of 22 germplasms of Sabah's traditional rice varieties were collected from different divisions of Sabah including WCD (n=9), SD (n=5) and ID (n=8). The length, width, thickness and colour of the rice seeds were determined. The size of the rice seeds were grouped into short (<6 mm), medium (between 6-7 mm) and long (>7 mm) based on their length. The shape of the rice seeds was determined based

on the length/width ratio and categorised into bold (ratio of 1.1-2.0), medium (ratio of 2.1-3.0) and slender (ratio>3.0) (JICA, 2013). The head rice recovery, moisture content and amylose content were determined according to previous approaches (Jindal & Siebenmorgan, 1987; Avaro et al., 2009; Kamruzzaman et al., 2012). Table 1 shows the characteristics of all Sabah's traditional rice varieties in this study. Genomic DNA from seeds was isolated using the cetyl trimethylammonium bromide (CTAB) method with slight modifications from previously described (Sharma et al., 2013) where CTAB was used instead of polyvinylpyrrolidone (PVP) and β-mercaptoethanol.

Table 1
Characteristics of Sabah's traditional rice varieties presented in this study

Rice Varieties	Length (mm)	Width (mm)	Thickness (mm)	Size[a]	Shape[b]	Colour[c]	Head Rice Recovery	Moisture Content	Amylose Content[d]
PBT02	7.40	1.84	1.30	LG	S	RB	78.57%	7.99%	IM
PBT06	6.30	2.79	1.59	MD	M	PB	74.29%	8.45%	H
PBT07	6.50	2.84	1.85	MD	M	PB	85.42%	8.15%	H
PBT08	6.00	3.00	1.90	MD	B	RB	85.42%	8.30%	L
PBT09	7.60	3.20	1.94	LG	M	B	81.82%	8.11%	IM
PBT10	6.70	2.72	1.45	MD	M	PB	80.00%	6.39%	VH
PBT11	6.20	2.79	1.59	MD	M	RB	96.00%	7.10%	IM
PBT12	6.30	3.07	1.82	MD	M	CW	87.10%	8.59%	IM
PBT13	5.30	1.88	1.32	ST	M	CW	75.56%	8.22%	VH
PBT14	7.20	2.52	1.82	LG	M	CW	84.85%	8.32%	H
PBT16	6.50	2.34	1.68	MD	M	PB	68.75%	6.28%	L
PBT17	6.80	2.12	1.35	MD	S	RO	91.30%	9.53%	IM
PBT18	6.20	1.91	1.44	MD	S	CW	86.36%	13.13%	IM
PBT19	6.80	2.98	1.64	MD	M	PB	91.67%	7.96%	L
PBT20	7.50	2.60	1.49	LG	M	CW	80.49%	8.67%	L
PBT21	7.20	2.46	1.70	LG	M	CW	88.00%	12.32%	H
PBT22	6.30	2.47	1.68	MD	M	CW	79.55%	8.68%	H
PBT23	5.70	2.09	1.26	ST	M	CW	90.91%	8.20%	VH
PBT24	7.20	2.18	1.52	LG	S	RO	86.84%	8.79%	H
PBT25	6.80	2.98	1.64	MD	M	RB	84.62%	8.54%	IM
PBT26	5.50	2.90	1.88	ST	B	CW	89.13%	6.61%	H
PBT27	6.90	2.12	1.74	MD	S	CW	82.93%	8.01%	L

[a]ST, short (length<6 mm); MD, medium (length between 6-7 mm); LG, long (length >7 mm).
[b]Based on length/width ratio. B, bold (ratio between 1.1-2.0); M, medium (ratio between 2.1-3.0); S, slender (ratio >3.0).
[c]RB, reddish brown; PB, purplish black; B, black; CW, creamy white; RO, reddish orange.
[d]L, Low (15-22% amylose content); IM, intermediate (23-26% amylose content); H, high (27-30% amylose content); VH, very high (>30% amylose content).

PCR Amplification Using RAPD Markers

A total of 22 RAPD primers were initially screened for the presence of bands. Out of them, only 11 were with reproducible amplification and were selected to perform genetic diversity analysis for all rice varieties (Table 2). PCR was carried out in a final 25 µL reaction volume containing 50 ng of template DNA, 1x PCR Buffer, 2.0 mM of $MgCl_2$, 0.2 mM of dNTPs mixture, 0.2 µM of each primer and 0.2 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif). The conditions of PCR were set at 1 cycle of initial activation for 4 min at 95°C, 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 40°C, extension of 2 min at 72°C, and 1 cycle of final extension for 2 min at 72°C in SpeedCycler^2 thermal cycler (Analytik Jena, Jena, Germany). Amplified PCR products were analysed using Fragment Analyzer™ (Advance Analytical Technologies, Ames, IA).

Table 2
List of 11 RAPD primers and genetic variation in 22 of Sabah's traditional rice varieties

Primer Name	Sequence (5'-3')	Range of Size (bp)	Total no. of Bands	No. of Polymorphic Bands	Percentage of Polymorphism (%)
OPA-01	CAGGCCCTTC	698-3000	9	9	100
OPA-02	TGCCGAGCTG	298-2951	8	8	100
OPA-03	AGTCAGCCAC	372-2131	8	8	100
OPA-04	AATCGGGCTG	431-3144	7	7	100
OPA-10	GTGATCGCAG	406-2738	9	9	100
OPA-12	TCGGCGATAG	1491-1848	1	1	100
OPA-13	CAGCACCCAC	447-2875	8	8	100
OPB-07	GGTGACGCAG	444-6104	9	9	100
OPB-10	CTGCTGGGAC	341-1870	7	7	100
OPB-12	CCTTGACGCA	278-2525	4	4	100
OPC-15	GACGGATCAG	305-3000	5	5	100
	Total		75	75	100

Genetic Diversity and Relationship Analysis

Each polymorphic band was scored as a binary code of 1 (presence) or 0 (absence). Jaccard's similarity matrix was calculated using DendroUPGMA online software (http://genomes.urv.cat/UPGMA/). GenAlEx ver.6.41 software (Peakall & Smouse, 2006) was used to calculate the genetic parameters including number of alleles (N_a), number of effective alleles (N_e), expected heterozygosity (H_e) and Shannon's information index (I). The same software was used to determine molecular variance (AMOVA) and perform the principal component analysis (PCA). Molecular Evolutionary Genetic Analysis 6 (MEGA6)

Software (Tamura et al., 2013) was utilised to construct a phylogenetic tree using the Neighbour Joining method with bootstrap replicates of 1000. In addition, the genetic differentiation index of PhiPT (ϕ_{st}) among divisions, Nei's genetic distance and Nei's genetic identity were also calculated using GenAlEx ver.6.41 software. The gene flow level (Nm) was determined based on the formula previously described (Slatkin & Barton, 1989).

RESULTS AND DISCUSSION

DNA-based molecular markers were reported as useful tools in the assessment of genetic diversity and elucidation of the relationship between different rice varieties (Ragunathanchari et al., 2000; Shivapriya & Hittalmani, 2006). Various molecular techniques to study genetic diversity are available including amplified fragment length polymorphism (AFLP) (Zabeau & Vos, 1993), restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), simple sequence repeats (SSRs) (Tautz, 1989) and RAPD (Williams et al., 1990). Among them all, the RAPD approach is the most inexpensive and rapid as it requires no information regarding the genome of the plant. In addition, it has been widely applied in rice genetic diversity studies (Rahman et al., 2007; Rajani et al., 2013; Abdul-razzak Tahir, 2014; Alam et al., 2014; Hasan & Raihan, 2015).

The characteristics of Sabah's traditional rice varieties in this study showed that more than half of the rice seeds were medium in size (59.09%) and shape (68.18%) (Table 1). The head rice recovery of the rice seeds was considered moderately high (ranging from 60.75% to 96.00%) but all had low moisture content (<14.00%). It is recommended to harvest rice at 18-24% moisture content to avoid fissuring of the seeds. The optimum milling potential for rice is at the moisture content of 14% wet weight basis (JICA, 2013). In addition, about half of the collected rice seeds (45.45%) were categorised with high to very high amylose content, making them less tender, dry when cooked and hard upon cooling (JICA, 2013).

In this study, all the amplified bands generated by 11 RAPD markers were analysed using Fragment Analyzer™ (Figure 1). A total of 75 bands were scored (Table 2). The number of bands produced ranged from 1 to 9 bands with the minimum number of one band produced by OPA-12 primer and the maximum number of 9 bands were produced by OPA-01, OPA-10 and OPB-07 primers. The size of bands produced ranged from 278 to 6104 bp. All 11 RAPD primers produced 75 polymorphic bands with 100% polymorphism. The high level of polymorphism generated by all RAPD markers in this study was dramatically higher compared to the results of previous studies conducted in Thailand (Kanawapee et al., 2011), Bangladesh (Hasan & Raihan, 2015), Iraq (Abdul-razzak Tahir, 2014) and India (Rajani et al., 2013). Guo et al. (2007) reported that geographic isolation may play an important role during the process of genetic diversification and variation. As Sabah is geographically isolated from the mainland of Asia, the significant high level

of polymorphism could be due to great intra-specific variation among the traditional rice varieties in different divisions of Sabah for better adaptation to environment changes and survival rate.

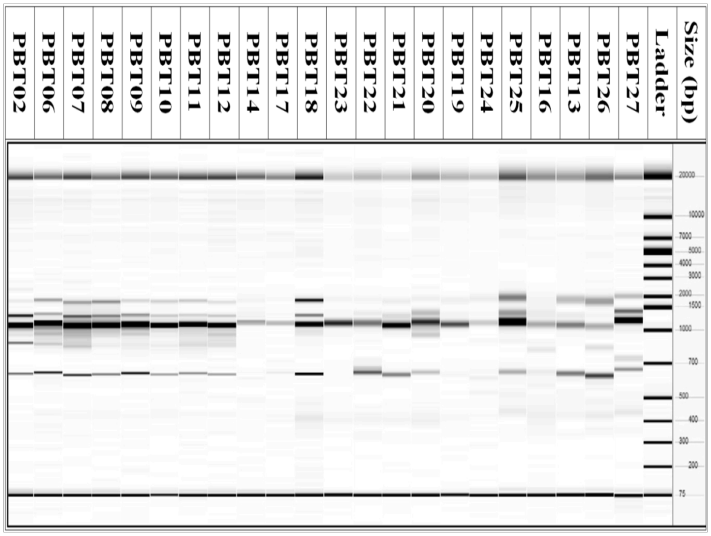

Figure 1. A representative banding profile of 22 different traditional rice varieties of Sabah using OPB-07 RAPD primer generated from Fragment Analyzer™

Our study showed that PBT10 and PBT11 rice varieties from WCD had the closest genetic relationship (Jaccard's similarity coefficient=0.864) (Table 3). On the other hand, PBT22 and PBT24 rice varieties from ID had the most distant genetic relationship (Jaccard's similarity coefficient=0.095). Jaccard's coefficient of similarity in this study ranged from 0.095 to 0.864, representing a high level of genetic variation among all Sabah traditional rice varieties. A high level of genetic variation of rice was also reported in different countries including in Thailand, with genetic similarity ranging from 0.64 to 0.94 (Kanawapee et al., 2011), India, with genetic similarity ranging from 0.47 to 0.81 (Rajani et al., 2013) and Bangladesh, with genetic similarity ranging from 0.101 to 0.911 (Hasan & Raihan, 2015).

Table 3
Similarity matrix of Sabah traditional rice varieties using Jaccard's similarity coefficient

	PBT02	PBT06	PBT07	PBT08	PBT09	PBT10	PBT11	PBT12	PBT13	PBT14	PBT16	PBT17	PBT18	PBT19	PBT20	PBT21	PBT22	PBT23	PBT24	PBT25	PBT26	PBT27
PBT02	1.000	0.735	0.517	0.607	0.712	0.596	0.635	0.593	0.510	0.491	0.490	0.392	0.698	0.611	0.654	0.623	0.446	0.426	0.109	0.400	0.377	0.537
PBT06		1.000	0.579	0.712	0.796	0.673	0.714	0.667	0.617	0.560	0.469	0.458	0.816	0.623	0.700	0.667	0.509	0.462	0.163	0.409	0.469	0.608
PBT07			1.000	0.759	0.709	0.660	0.667	0.655	0.491	0.527	0.393	0.407	0.667	0.643	0.596	0.569	0.410	0.439	0.143	0.308	0.444	0.571
PBT08				1.000	0.755	0.851	0.816	0.765	0.620	0.482	0.453	0.500	0.741	0.685	0.636	0.636	0.491	0.500	0.170	0.396	0.426	0.706
PBT09					1.000	0.720	0.796	0.780	0.600	0.547	0.520	0.451	0.824	0.698	0.679	0.648	0.500	0.455	0.174	0.404	0.490	0.593
PBT10						1.000	0.864	0.804	0.644	0.434	0.429	0.478	0.673	0.680	0.596	0.596	0.472	0.480	0.200	0.395	0.458	0.702
PBT11							1.000	0.771	0.652	0.444	0.469	0.458	0.712	0.686	0.604	0.604	0.481	0.462	0.163	0.409	0.500	0.640
PBT12								1.000	0.638	0.463	0.460	0.511	0.698	0.673	0.593	0.593	0.473	0.453	0.186	0.370	0.460	0.660
PBT13									1.000	0.400	0.561	0.590	0.588	0.560	0.510	0.571	0.469	0.478	0.167	0.350	0.524	0.682
PBT14										1.000	0.404	0.524	0.566	0.538	0.519	0.549	0.480	0.400	0.100	0.217	0.294	0.407
PBT16											1.000	0.568	0.481	0.574	0.521	0.521	0.388	0.422	0.188	0.351	0.395	0.522
PBT17												1.000	0.500	0.500	0.511	0.511	0.571	0.512	0.161	0.231	0.349	0.511
PBT18													1.000	0.655	0.765	0.731	0.545	0.500	0.170	0.396	0.426	0.582
PBT19														1.000	0.673	0.776	0.491	0.529	0.182	0.362	0.423	0.615
PBT20															1.000	0.755	0.620	0.540	0.159	0.340	0.460	0.596
PBT21																1.000	0.620	0.571	0.186	0.340	0.431	0.596
PBT22																	1.000	0.532	0.095	0.184	0.333	0.472
PBT23																		1.000	0.200	0.317	0.422	0.480
PBT24																			1.000	0.217	0.152	0.143
PBT25																				1.000	0.282	0.429
PBT26																					1.000	0.429
PBT27																						1.000

When stratified to different divisions, the estimated allele frequency with number of different alleles (N_a) and effective alleles (N_e) was highest in ID (N_a=1.693±0.080, N_e=1.505±0.043), while the lowest in SD (N_a=1.413±0.093, N_e=1.366±0.044) (Table 4). Among all the three divisions, Sabah's traditional rice varieties in ID showed the highest genetic diversity (I=0.434±0.029, H_e=0.239±0.013) and percentage of polymorphic loci (82.67%), whereas the lowest genetic diversity (I=0.315±0.034, H_e=0.213±0.024) and percentage of polymorphic loci (58.67%) were in WCD, which further supported that PBT22 and PBT24 from ID had the lowest Jaccard's similarity coefficient while PBT10 and PBT11 from WCD had the highest Jaccard's similarity coefficient. The AMOVA analysis revealed that the total genetic differentiation coefficient of the three divisions was 0.145, indicating that the genetic differentiation among the divisions was relatively small (Table 5). The genetic differentiation of Sabah's traditional rice varieties was more likely to occur within divisions (85%) rather than among divisions (15%), and a similar finding was obtained by a study conducted in China (Hu et al., 2014).

Table 4
Genetic parameters of Sabah's traditional rice varieties in different divisions

Division	PPL (%)	N_a	N_e	I	H_e
WCD	58.67	1.427±0.087	1.372±0.045	0.315±0.034	0.213±0.024
SD	61.33	1.413±0.093	1.366±0.044	0.321±0.033	0.214±0.023
ID	82.67	1.693±0.080	1.505±0.043	0.434±0.029	0.291±0.021
Overall	100.00	1.511±0.051	1.414±0.026	0.357±0.019	0.239±0.013

WCD, West Coast Division; SD, Sandakan Division; ID, Interior Division; PPL, percentage of polymorphic loci; N_a, number of different alleles; N_e, number of effective alleles; I, Shannon's information index; H_e, expected heterozygosity

Table 5
AMOVA analysis of Sabah's traditional rice varieties in different divisions

Source	*df*	SS	MS	Variance Component	Variation (%)	PhiPT	*p*
Among Division	2	47.107	23.553	1.807	15.00	0.145	<0.001
Within Division	19	202.439	10.655	10.655	85.00		<0.001
Overall	21	249.545		12.462	100.00		

df, degree of freedom; SS, sum of squares; MS, mean of sum of squares

The phylogenetic analysis classified Sabah's traditional rice varieties into four groups, with close relationship found between SD and ID as revealed in Group 2 to 4 (Figure 2). Although some rice varieties in SD and ID were grouped together with WCD in

Group 1, the PCA revealed no evidence of genetic overlapping of rice varieties in WCD to either SD or ID (Figure 3). From the PCA analysis, rice varieties in SD and ID exhibited scattered distribution and presented overlapped rice varieties. This indicated that rice varieties in SD and ID contained the richest genetic information and were closely related, as supported by a low PhiPT genetic differentiation index (0.022) and high gene flow (N_m) value (11.114) (Table 6) as well as a low Nei's genetic distance (0.104) and high Nei's genetic identity (0.901) (Table 7) between SD and ID. The accuracy of the phylogenetic analysis and PCA in this study can be improved by including the environmental changes and human activity over time as these two factors may influence the genetic diversity of plants (Tilman & Lehman, 2001; Helm et al., 2009).

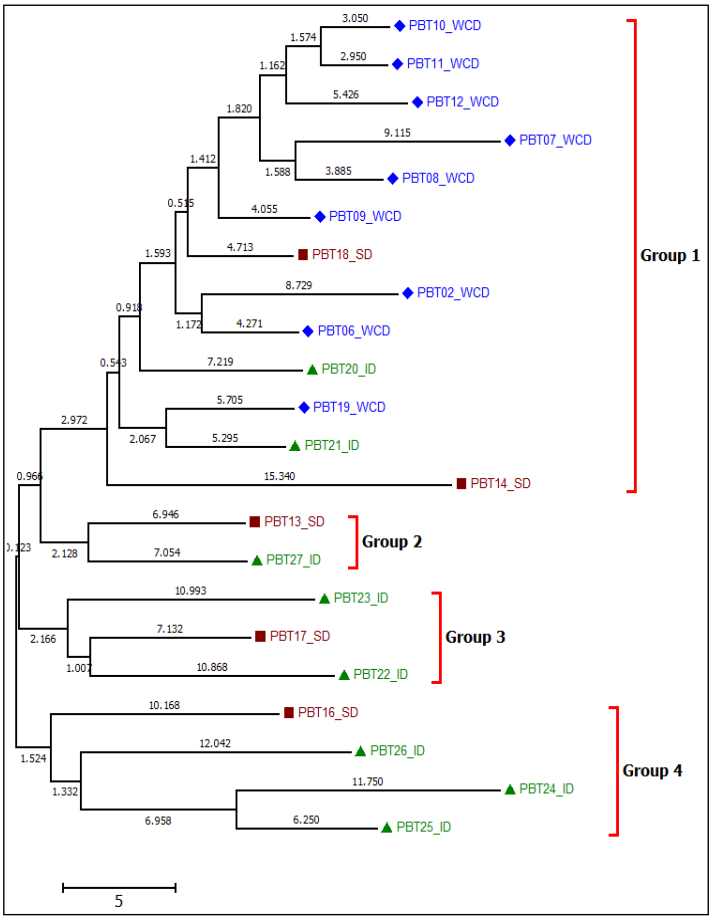

Figure 2. Phylogenetic tree of the traditional rice varieties of Sabah in different divisions constructed using the Neighbour Joining method with 1000 bootstrap replicates in MEGA

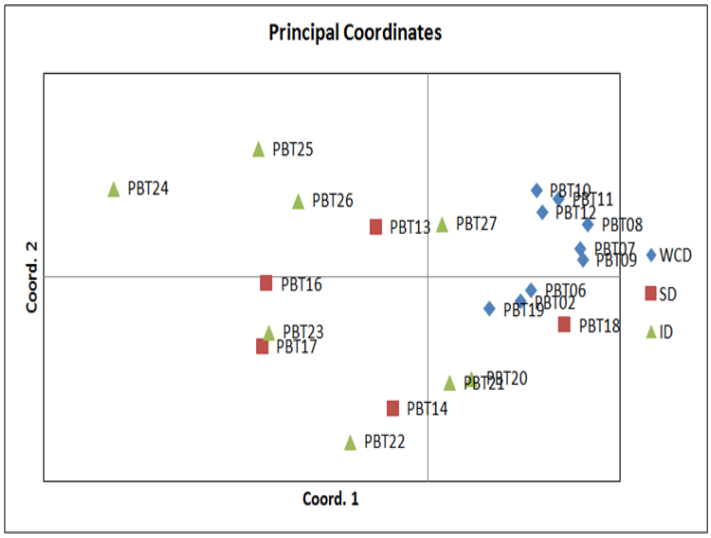

Figure 3. Principal component analysis (PCA) of genetic diversity among the traditional rice varieties of three divisions of Sabah including West Coast Division (WCD), Sandakan Division (SD) and Interior Division (ID)

Table 6
Genetic differentiation of PhiPT (lower left) and gene flow (N_m) (upper right) analysis among Sabah's traditional rice varieties in different divisions

Division	WCD	SD	ID
WCD	-	1.059	1.073
SD	0.191	-	11.114
ID	0.189	0.022	-

WCD, West Coast Division; SD, Sandakan Division; ID, Interior Division

Table 7
Nei's genetic distance (lower left) and Nei's genetic identity (upper right) of Sabah's traditional rice varieties in different divisions

Division	WCD	SD	ID
WCD	-	0.862	0.851
SD	0.149	-	0.901
ID	0.161	0.104	-

WCD, West Coast Division; SD, Sandakan Division; ID, Interior Division

CONCLUSION

In summary, genetic diversity assessed using 11 RAPD markers in this study revealed a high level of polymorphism and genetic variation among all Sabah's traditional rice varieties. The genetic differentiation of Sabah's traditional rice varieties was more likely to occur within divisions rather than among divisions. Sabah's rice varieties in ID had the highest genetic diversity and polymorphic loci, and were closely related to rice varieties in SD while distanced to WCD. Therefore, cross-breeding of Sabah's traditional rice varieties in WCD with those in either SD or ID is recommended to enhance the genetic diversity of rice varieties. As Sabah's traditional rice plantations are mainly grown by conventional farmers who do not have a proper recording system and comprehensive

information of genetic diversity in their rice varieties, information on genetic diversity and the relationship between Sabah's traditional rice varieties in this study may provide insight for local breeders into selective breeding and cross-breeding programmes for rice yield increment.

ACKNOWLEDGEMENT

The authors would like to thank the Sipitang District Council, the Department of Agriculture Sipitang, the Department of Agriculture Tongod and local farmers for providing the seeds for this study. This study was supported by the Ministry of Higher Education Malaysia under the ERGS Grant Scheme (ERGS0031-STG-1/2013).

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