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Analysis of Sarawak Bario Rice Diversity Using Microsatellite Markers

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Abstract: Problem statement: There are numerous claims on varieties of Bario rice cultivars in Sarawak. Many of them might be synonyms or homonyms. This preliminary study was to differentiate Bario Rice cultivars using microsatellite molecular markers. **Approach:** Microsatellite marker panels distributed on all 12 chromosomes on rice genome were employed to study 8 Bario rice cultivars in Sarawak. **Results:** Current study indicated that the microsatellite panel were able to differentiate the 8 Bario rice cultivars. A total of 31 alleles were generated at 12 polymorphic microsatellite loci among the cultivars using the selected polymorphic SSR primer pairs with an average of 2.6 alleles per locus. Average PIC value obtained was 0.5204. An UPGMA dendrogram based on SSR polymorphism indicated high variation among the rice varieties with the coefficient ranging from 0.16 and 0.92. Genetic diversity determination using cluster analysis showed differentiation of rice cultivars into 2 major groups and several sub-groups. **Conclusion:** The SSR markers were useful for differentiating the genotypes and assessment of genetic relationships due to its reproducibility and to multiallelic, codominant and informative properties.

Key words: Microsatellite markers, genetic diversity, Bario rice cultivars, Sarawak

INTRODUCTION

Bario rice cultivars are traditional rice of the land of the Hornbill (Sarawak) which are planted by local ethnic community in the Highlands. Bario rice has earned its name as one of the finest rice grains in the world due to its soft texture, fine elongated grains with its exquisite taste and aroma[1]. Due to the gaining popularity of Bario rice in the market, there has been irresponsible exploitation of the brand name Bario with other rice cultivars which are actually cheaper and of lesser quality than the genuine Bario rice. Even though there are numerous claims on varieties of Bario rice cultivars in Sarawak, many of them might be synonyms or homonyms. Hence the need to develop the DNA fingerprinting profile or genotype of genuine Bario rice cultivars by using molecular markers such as microsatellite markers, random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphic DNA (RFLP).

Microsatellites or Simple Sequence Repeats (SSR) have become major molecular markers for a wide range of studies in plants and animals after its emergence as a Polymerase Chain Reaction (PCR)-based genetic marker[2]. One of the well established features of SSR loci is their hypervariability, which is associated with the expansion potential of the SSR motif itself. This feature, in combination with the ease of PCR amplification, the co-dominant profiles and the potential for automation, has contributed to the widespread use of these markers in a range of genetic studies. SSR has been widely used in various plants and in rice alone it has been used to fingerprint accessions, analyze diversity, identify introgressions in interspecific crosses, trace pedigrees, locate genes and quantitative trait loci on rice chromosomes and in marker assisted selection[3].

The primary objective of this study was to differentiate 8 Bario rice cultivars in Sarawak and to determine their genetic diversity.

MATERIALS AND METHODS

Plant material: Seeds of Bario rice cultivars used for this study were either collected from farmers in the southern divisions of Sarawak or obtained from the Department of Agriculture Sarawak (Table 1). DNA was extracted by bulking the leaves harvested using "GF-1 Plant DNA Extraction Kit" from Vivantis Technologies.

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Table 1: List of Bario rice cultivars analyzed

No	Rice cultivar	Cultivar code	Source
1	Bario	B.	Department of Agriculture Sarawak
2	Bario-2	B.2	Department of Agriculture Sarawak
3	Bario Banjal	B. Banjal	Tatau
4	Bario Chelum	B. Chelum	Department of Agriculture Sarawak
5	Bario Halus	B. Halus	Tatau
6	Bario Lowland	B. Low (M)	Meradong
7	Bario Lowland	B. Low (S)	Kampung Bunan, Serian
8	Bario Sederhana	B. Sdrhn	Tatau

Selection of microsatellite markers: Microsatellite marker panels well distributed on all 12 chromosomes on rice genome were used in this study. These panels were chosen based on ability to differentiate the Sarawak rice cultivars in the preliminary screening performed in our laboratory.

Polymerase Chain Reaction and Evaluation: Protocols for Polymerase Chain Reaction (PCR) with Taq DNA polymerase was based on the protocol provided with the "DNA Amplification Kit" from Vivantis Technologies. PCR was performed in 25 µL reaction mixture containing 1 µL of extracted plant DNA, 0.4 µM of each primer, 200 µM dNTP for each nucleotide, 50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$ and 2.0 units Taq DNA Polymerase (Vivantis) in a 0.2 mL PCR tube. Amplification was carried out using BIOER XP thermocycler as follows: Initial denaturation at 95°C for 3 min; denaturation at 94°C for 30 sec, primer annealing for 30 sec where the annealing temperature varies with the type of primer used; elongation at 72°C for 1 min, follow by 32 cycles of denaturation, primer annealing, elongation and 5 min at 72°C for final elongation. Negative and positive control was incorporated in each set of PCR amplification conducted to verify the absence of contamination during PCR and its efficiency[4].

PCR products (5 µL) were resolved on polyacrylamide gel for higher resolution using Mini-PROTEAN 3 Cell gel casting apparatus (BIORAD, USA) to achieve high resolution of the bands[5]. The sizes of amplified fragments were estimated with FlourChem 5500 using VC 100 bp plus DNA ladder (Fermentas, Lithuania) and Ultra Low Range DNA Ladder (Vivantis Technologies) as the size standards. SSR amplified products with expected product size above 150 bp was subjected to 5% polyacrylamide gel electrophoresis at 80 volts for 60 min. Meanwhile for expected product size below 150 bp, 6% polyacrylamide gel was used with 80 volts for 50 min. After staining in 0.5 µg mL^{-1} ethidium bromide for 20 min and de-stained, PAGE gels were visualized under ultraviolet light using the procedure described in Sambrook and Russell[6] and documented in gel documentation system[7] (Alpha Innotech, USA).

Molecular analysis: Amplified alleles were visualized as bands on gels. Only clear and apparently unambiguous bands present on the polyacrylamide gel were scored[8]. Using the allele sizes obtained with each SSR marker for 8 Bario rice cultivars the input matrix for genetic analysis in binary format was prepared. Nei's standard genetic distances were computed using rectangular matrix formed from allelic frequency computations derived using NTSYSpc (version 2.2).

Scoring of Main Allele Band: The size in nucleotide base pairs (bp) of allele band with highest intensity for each SSR marker was scored as main allele band and determined based on its migration relative to molecular-weight size marker[9]. An allele that was observed in only one of the 14 rice cultivars was determined as rare or unique allele[10,11].

Verification of SSR Primers Used For Analysis of Genetic Relationship: To ensure the reproducibility of the 12 selected highly polymorphic SSR primers and result verification purpose, PCR amplification of different batches of DNA sample were performed.

Data analysis: Markers were scored based on the band pattern generated from the gel imaging system for the presence or absence of the corresponding band among the genotypes. Using the binary coding system, '1' indicating the presence of clear and unambiguous bands and '0' indicating the absence of bands, the SIMQUAL subprogram in NYTSYSpc was used to generate the similarity sequence. The data obtained from SIMQUAL was later used to estimate the genetic relationships among the 8 Bario rice cultivars. The data gathered were subjected to cluster analysis using NYTSYSpc (version 2.2) to generate a dendrogram by Unweighted Pair Group with Arithmetic Averages (UPGMA) method[8,12-14].

Polymorphism Information Content (PIC) was calculated for each marker using the following equation:

$$PIC_i = 1 - \sum_{j=1}^{n} Pij^2$$

where, Pij is the frequency of the j^{th} allele for ith marker and summation extends over n alleles. Cluster analysis

was performed using NTSYSpc. The resulting distance matrix data was used to construct the dendrogram using the un-weighted pair-group method with arithmetic average (UPGMA).

RESULTS

Number of alleles: Using 12 SSR primers with polymorphic loci which were fully distributed on all the rice genomes that had been pre-screened, a total of 31 alleles were detected among the 8 Bario rice cultivars (Table 2 and Fig. 1). The average number of alleles per locus was 2.6 alleles with 2 alleles in 6 markers, 3 alleles in 5 markers and 4 alleles in 1 marker. The overall size of amplified products ranged from 79 bp (RM413) to 236 bp (RM404). A total of 2 unique alleles were detected at 4 SSR loci (RM11, RM404, RM271 and RM206). The SSR primer pairs used for the genetic diversity analysis, the number of alleles for each SSR locus and PIC values are shown in Table 2.

Polymorphism of SSR markers: All of the 12 SSR primer pairs used for analysis of genetic diversity and relationship in this study generated polymorphic bands among the cultivars. The PIC value for the SSR loci ranged from 0.3750 to 0.6563 with an average of 0.5204 (Table 2). The highest PIC value was observed for primers RM 204 and RM271 (0.6563). The PIC values for the remaining SSR loci were 0.5938 or lower.

Cluster analysis: The input matrix for genetic analysis among the rice cultivars were prepared from the scoring of main alleles obtained. A dendrogram was generated by UPGMA to show the genetic relationships of the Bario rice cultivars studied. The cluster analysis generated showed a significant genetic variation among the rice cultivars studied, with similarity coefficients ranging between 0.16 and 0.92. The dendrogram revealed 2 distinct groups (I and II) at the similarity coefficient of 0.16 (Fig. 2).

The first group (Group I) consists of 6 Bario rice cultivars, namely Bario, Bario Chelum, Bario-2, Bario Halus, Bario Lowland (Meradong) and Bario Sederhana. The similarity coefficients ranged between ~0.39 and 0.92, while the second group (Group II) consists of only Bario Banjal and Bario Lowland (Serian), with the genetic coefficients 0.667. Group I was further sub-divided into several sub-groups.

In group I, there were 2 sub-groups forming clusters of loosely related varieties. The first sub-group consists of Bario, Bario-2 with similarity coefficient of 0.833. Another cluster comprised of Bario Chelum, Bario Halus and Bario Lowland (Meradong). Bario Sederhana was quite diverted from the rest of the cultivars. Bario Halus and Bario Lowland (Meradong) were highly similar with the similarity coefficient of 0.917.

SSR markers in rice studies: Presences of stutter bands were detected in this study. Stutter band are minor products amplified in PCR that have lower intensity than the main allele and normally lacks or has extra repeat units[11]. Although stutter bands were very common and more serious in di-nucleotide SSR, it was observed in tri-nucleotide SSR such as in RM 19 as well (Chromosome 12) and in tandem repeat sequence such as RM 316 (Chromosome 9) but was less serious.

Table 2: Allele variation and PIC values for SSR loci identified in 8 Bario rice cultivars

Chromosome position	SSR marker	Expected PCR product size (bp)	No. of alleles	No. of unique alleles	PIC value
1	RM1	113	2.0		0.4688
2	RM240	132	2.0		0.4688
3	RM489	271	2.0		0.3750
4	RM252	216	2.0		0.4688
5	RM413	79	3.0		0.4939
6	RM204	169	3.0		0.6563
7	RM11	140	3.0	1	0.5938
8	RM404	236	3.0	1	0.5938
9	RM316	192	2.0		0.4688
10	RM271	158	4.0	1	0.6563
11	RM206	147	3.0	1	0.5313
12	RM19	226	2.0		0.4688
Total			31.0	4	6.2444
Mean			2.6		0.5204

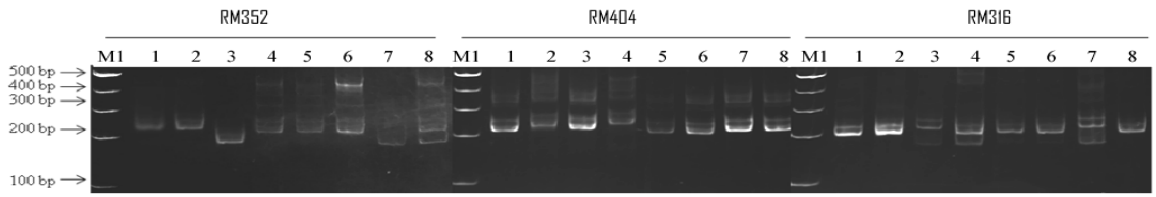

Fig. 1: Polymorphic SSR bands of 8 Bario rice cultivars amplified by RM252, RM404, RM316 (M1-VC 100 bp DNA Ladder)

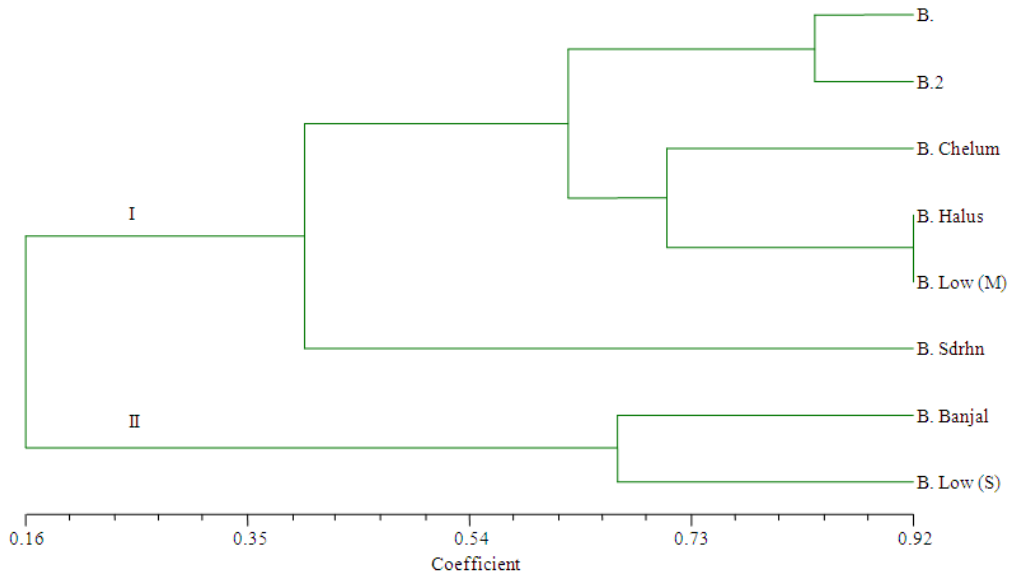

Fig. 2: Dice' similarity coefficient based on 12 SSR makers showing the relationships among 8 Sarawak Bario rice cultivars (Dendrogram derived from UPGMA cluster analysis)

This was in agreement with Cho *et al.*[15], where SSR loci with di-nucleotide repeat motifs tended to detect greater number of alleles than with tri-nucleotide and tandem repeats sequence. Based on the study of Olejniczak and Krzyzosiak[16], stutter bands were produced by the slippage of the polymerase amplification and the factors that influence the proportion of stutter band to the main allele were the repeat number, number of PCR cycles, length and the characteristics of the repeat sequence.

DISCUSSION

Polymorphism in SSR markers: Highest PIC values were observed for SSR primers RM204 and RM271 (0.6563). PIC value is reflection of allele diversity and frequency among the cultivars. The higher the PIC value of a locus, the higher the number of alleles detected. This observed pattern was consistent with the findings of Lapitan *et al.*[17] According to Akkaya and Buyukunal-Bal[4], high PIC value can be attributed to the use of more informative markers. The PIC value for the remaining SSR loci were all below 0.65 with an average of 0.4875.

From the results, a total of 31 alleles were detected among the 8 Bario rice cultivars (Table 2). The average number of alleles per locus was 2.6 alleles with a range of 2 to 4 alleles (RM204). Jalaluddin *et al.*[18] studied on 22 cultivars with 5 SSR markers also reported a range of 2-4 alleles which is similar to this study. On a per locus basis the average of 2.6 alleles were comparable with the average of 2.0-5.5 alleles per locus for various classes of SSRs reported by Cho *et al.*[15] This is significantly lower than the average number of alleles reported by other researchers[10,19,20]. However, these authors used much larger numbers of rice cultivars (176, 92, 33 and 38 cultivars respectively) and SSR loci (24, 19, 25 and 111 SSR, respectively).

According to Li *et al.*[21], polymorphism in the SSR could also be due to changes in the SSR region itself, caused by the expansion or contraction of SSRs, or interruption.

Intra and Inter-Cultivar Variation, Synonyms and Homonyms: There were cases of rice cultivars with the same name (Bario Lowland) but collected from different locations that do not exhibit the same band pattern and were not clustered together. The probable explanation was that there were cases of mislabeling during the establishment of rice germplasm collection or that the cultivars came from different source but given the same name by the farmers.

The rice itself is a self pollinating plant; however there is presence of heterozygosity. According to Garland *et al.*[22], intra cultivar polymorphism was due to presence of heterozygous sample. They also stated heterogeneity may be explained by a high mutation rate where the alleles were unstable within cultivar if the mutation rate was sufficient. Jalalludin *et al.*[18] reported that heterogeneity might be due to use of many rice

varieties for cultivation by the farmers and the production of the seeds by the farmers themselves could enhance the varietal mixtures and promoting natural cross pollination among cultivars in surrounding fields to maintain a stable yield.

An example of synonyms in this study was the grouping of Bario Halus and Bario Lowland (Meradong). These cultivars probably came from the same source but were given different names by the farmers. Intra-cultivar variation was observed in some cultivars with the same name which was clustered to a distinct group such as Bario Lowland (Meradong) and Bario Lowland (Serian). Omrani *et al*.[23] also reported similar cases which may be due to homonyms and misidentification during germplasm collection. The unexpected variation is also most likely due to mass selection and selection of the rice seeds as a mixture representing acceptable properties within a cultivar[4].

Genetic Relationship among the Rice Cultivars: Dendrogram obtained using UPGMA method revealed cultivars that are derivatives of genetically similar type clustered together which explains the relationship among the 8 Sarawak Bario rice cultivars. The coefficient ranging between 0.16 and 0.92 indicated a great variation among the rice cultivars[12]. Chakravathi and Naraveni[24] state that the larger range of similarity values for cultivars revealed by SSR markers provides greater confidence for the assessment of genetic diversity and relationship, which can be used in future breeding programs and the practical approaches developed is also useful in DNA fingerprinting.

Bario and Bario-2 are considered to be genetically closely related and clustered together with a genetic similarity of 0.833. High degree (0.917) of genetic similarity among Bario Halus and Bario Lowland (Meradong) was obtained in this study. Such cases were probably due to the selections made by farmers from a single land race[25] or attributed to several genotypes in the development of traditional Bario rice planted by the ethnic community in Sarawak. According to Chakravarthi and Naravaneni[24] high similarity coefficients indicates cultivars belonging to similar genetic background. This scenario could be caused by occasional outcrossing events that may occur spontaneously or selectively between the cultivars[23].

According to Tu *et al*.[12] in the distribution of genetic diversity and relationship of Yunnan rice germplasm, the cultural habits of exchanging seeds among farm families, villages or regions might have contributed to the enhancing of rice genetic diversity within the region by adding different genotypes to the local rice gene pool. Such practices by the locals in Sarawak may well have aided in promoting the genetic diversity of rice in Sarawak.

CONCLUSION

The microsatellite marker panel used for the analysis of 8 Bario rice cultivars in Sarawak was informative and useful for differentiating the studied cultivars. SSR marker RM204 and RM271 showed the highest PIC value (0.6563), while the average PIC value was 0.5204. From this study, it was found that rice cultivars with the same name (Bario Lowland) but collected from different location may not necessary be the same cultivar. This probably reflects the diverse practices with respect to cultural aspects, seed saving and seed exchange that could have contributed to the genetic diversity of Bario rice germplasm in Sarawak.

The identification and differentiation of the Sarawak Bario rice cultivars studied using microsatellite markers was achieved. The markers reported here is of value in ongoing efforts to construct a database in breeding programs and characterization of other Bario rice cultivars that are not included in this study.

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