Evaluation of Genetic Diversity of Local-Colored Rice Landraces Using SSR Markers

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Abstract. Analysis of genetic diversity of 90 Vietnamese local-colored rice accessions was carried out using 40 SSR markers. The numbers of polymorphic alleles ranged from 3 to 12 alleles per locus and average of 7.1 alleles per locus. The similarity coefficients of the rice landraces fluctuated from 0.76 to 0.93; at a genetic correlation level of 0.78. Ninety accessions of rice landraces were divided into five groups based on analysis of genetic relationships. The results have indicated that 11 markers included M250, RM302, RM10926, RM208, RM227, RM17231, RM23251, RM5647, RM1376, RM339 and RM228 which gave the unique allele. These markers can be used effectively for genetic diversity of colored rice and provided a specific database and useful materials for landraces identification, local germplasm conservation for further colored rice improvement on rice quality via rice breeding programs in Vietnam.

Introduction

Colored rice (Oryza sativa L.) is a rich sources of fat-soluble bioactive components which has high concentrations of protein, total essential amino acids, antioxidant compounds, vitamin B1 and other minerals to compare with the common rice [1-3]. Vietnam is known to be the center of rice diversity germplasm. Rice landraces are much diversified and played a key role in rice breeding for rice quality improvement in this country [4]. Among rice germplasm, local-colored rice landraces are abundant in this country, therefore, it should be exploited to develop commercial rice of high quality and rich medicinal value via rice breeding program.

The recent advances in terms of molecular biology such as PCR, DNA sequencing and data analysis technologies have significantly contributed to analysis and evaluation of rice genome. Among these mentioned technologies, the PCR method has emerged as a useful and popular technique that can be used for analysis of rice genome, particularly in the estimation of rice genetic diversity [5]. SSR markers are able to estimate genetic diversity between cultivars e.g. between parents of genepool or between plants extracted from a population or between populations. Microsatellites are more powerful for the identification of within cultivar variation.

Based on the characterization and evaluation of rice genetic diversity, plant breeders have been perceived the necessary information to identify initial materials for rice breeding via mutation, hybridization to produce landraces with high yield and good quality as well as resistance to biotic and abiotic stresses. Parallel to breeding, information gained from analysis of rice genetic diversity are also very useful for sustainable conservation of plant genetic resources for food and sustainable agriculture.

Recent research on the genetic diversity of colored rice mostly focused on anthocyanin content [6], Fe and Ze content [7, 8], or genetic diversity using both SSR markers and morphological characters [9]. The objective of this study was to evaluate the genetic diversity of 90 local - colored rice landraces in Vietnam using SSR markers. This study will provide useful information on genetic diversity among the colored rice landraces for further rice breeding.
Materials and Methods

Plant Materials

The plant materials included 90 diverse rice accessions were kindly provided by the Plant Resources Center. The rice landraces were collected from some different districts and provinces in the Central and the North of Vietnam as shown in Table 1. In order to evaluate the genetic diversity of rice landraces, 40 SSR primers were selected and used as listed in Table 2. These primers were selected to cover the rice genome with a representation for the 12 chromosome and based on the published paper [10].

Methods

- **DNA extraction:**

  Total genomic DNA extraction from leaves of three weeks old seedlings were carried out following the CTAB method [11].

- **PCR assay:**

  Polymerase chain reaction was implemented in the Veriti 96 well Thermal cycler. The total reaction solution was 20 µl including 2 µl PCR buffer 10x; 1.6 µl dNTP 2.5mM; 1.4 µl primer (forward and reverse primer) 25ng/µl; 0.1 µl green Taq (5U/ µl) and 5 µl DNA (5ng/µl). A programmable thermal controller set for 35 cycles in which each cycle is for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were electrophoresed on 8% polyacrylamide gel, and analyzed under the UV trans-illuminator for DNA detection.

  Band scoring: The gels were scored for computer analysis on the basis of the presence and absence of the amplified products. If a product was present in a genotype, it was designated as ‘1’ and if absent, it was designated as ‘0’.

- **Data analysis**

  The genetic similarity was analyzed by using Jaccard similarity coefficient in NTSYSpc 2.11X software using the method of Rohlf [12]. The genetic distance was calculated using the method of Nei [13]. Polymorphic Information Content (PIC) was calculated applying the methodology of Mohammad [14].

| No | Name of rice landrace | Accession number | Origin | No | Name of rice landrace | Accession number | Origin |
|----|------------------------|------------------|--------|----|------------------------|------------------|--------|
| 1  | Beo cu                 | GBVN001912       | Trang Dinh, Lang Son | 46 | Khau ho he              | GBVN0008678     | Tuong Duong, Nghe An |
| 2  | Pe le chua             | GBVN002013       | Than Uyen, Lao Cai   | 47 | Khau cam co             | GBVN008693      | Tuong Duong, Nghe An |
| 3  | Plei ha                | GBVN002019       | Than Uyen, Lao Cai   | 48 | Ple lau sang            | GBVN008713      | Bac Yen, Son La     |
| 4  | Nep cam Nuong          | GBVN002022       | Than Uyen, Lao Cai   | 49 | Ple sang loi            | GBVN008717      | Bac Yen, Son La     |
| 5  | Bao dam                | GBVN002024       | Than Uyen, Lao Cai   | 50 | Khau cam ky             | GBVN008755      | Phu Yen, Son La     |
| 6  | Nep cam den            | GBVN002056       | Bao Thang, Lao Cai   | 51 | Lo cam                  | GBVN008770      | Phu Yen, Son La     |
| 7  | Khau pet Lanh          | GBVN002093       | Yen Chau, Son La     | 52 | Piao co cam             | GBVN009446      | Da Bac, Hoa Binh    |
| 8  | Ne diem                | GBVN002102       | Yen Chau, Son La     | 53 | Khau lech               | GBVN009914      | Song Ma, Son La     |
| 9  | Khau pe                | GBVN002468       | Mai Son, Son La      | 54 | Ngo hieng               | GBVN012319      | Ky Son, Nghe An     |
| 10 | Khau lech 1            | GBVN002509       | Dien Bien, Lai Chau  | 55 | Khau lech               | GBVN012352      | Dien Bien           |
| 11 | Khau lech 2, dang 2    | GBVN002515       | Dien Bien, Lai Chau  | 56 | Khau tang san cha       | GBVN012565      | Da Bac, Hoa Binh    |
| 12 | Na ple la              | GBVN003562       | Mu Cang Chai, Yen Bai| 57 | Khau cao lan danh       | GBVN012593      | Da Bac, Hoa Binh    |
| 13 | Khau lo                | GBVN003918       | Quynh Nhai, Son La   | 58 | Blau ca dayk            | GBVN012983      | Bac Yen, Son La     |
| 14 | Pe lanh                | GBVN003921       | Thuan Chau, Son La   | 59 | Bie chong la            | GBVN013001      | Bac Yen, Son La     |
Table 2. List of 40 SSR primers used in this study

| No. | Primer | Chr | Annealing temp (°C) | PCR products (bp) | Forward Sequence | Reverse Sequence |
|-----|--------|-----|---------------------|-------------------|------------------|------------------|
| 1   | RM 250 | 2   | 55                  | 153               | GGTCTAACACAAAGCTGATCA | CATGGAAGCCCTCCCCAGCAG |
| 2   | RM 270 | 12  | 55                  | 108               | GCCTGGTGTCTTCTAAAATC | TCAGGCGAGCATCAGCGAG |
| 3   | RM 302 | 1   | 55                  | 156               | CTTATGTCATCTACCATCAC | ATTCGGAAGATGGAAATCTGC |
| 4   | RM 3825 | 1   | 55                  | 147               | AAAGCCCCAAAAGCGTAGT | GTGAAACTCTGGGTTGTCG |
| 5   | RM 201 | 9   | 55                  | 158               | CTCTGTTTATTCATTACAGTACC | CTACACTCTCCTTCTAGACCGATA |
| 6   | RM 1359 | 4   | 55                  | 170               | AACGAATTCTATTGTCGTC | TTCTTCTATTCAATTCG |
| 7   | RM 23251 | 8   | 55                  | 219               | TCCGATCTCCTACGGGATGG | ATGTTGGAAGGCTCATCTGG |
| 8   | RM 27027 | 8   | 55                  | 182               | GTTGCTGGCTGACCTCACAATGG | GTACGCCCTCCTGATTACGG |
| 9   | RM 6836 | 6   | 55                  | 240               | GGTGCGCATATGGGCTGATTGGA | GATACGCCCTTCTAGGCAAA |
| 10  | RM 6314 | 4   | 50                  | 169               | GATTTGCTGGTTGCAGTAG | GGGTTACAGGAGGAAATCTGC |
| 11  | RM 227 | 3   | 55                  | 106               | ACCTTTTCGTACAAAAAGCAG | GATTTGGAAGAAAGAAGCC |
| 12  | RM 21969 | 7   | 55                  | 99                | AGTTTCCCTCTCATTCCTTTTAGG | ACACAGAAGACTACAGGACGAGCC |
| 13  | RM 20589 | 6   | 55                  | 263               | CATGTATTGTGGGCACGTACC | ACCTTTCTTGCTCTTCTGG |
| 14  | RM 20590 | 6   | 55                  | 243               | TTTGTATGAGACCTTTCTCTTACC | GCCTGCGGCTTATCTACG |
| 15  | RM 347 | 3   | 55                  | 207               | CACCTTACATTTAACCAGGC | TCCGGCAAGGGATACCGG |
| 16  | RM 122 | 5   | 55                  | 277               | GAGTCGATGTAATGTCATCGTGC | GAAGGGAAGTTACGCTTGGT |

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| No | Seed coat color | Number of accession | Percentage (%) |
|----|-----------------|---------------------|----------------|
| 1  | Brown           | 13                  | 14.4           |
| 2  | Red             | 30                  | 33.3           |
| 3  | Variable purple | 42                  | 46.7           |
| 4  | Purple          | 5                   | 5.6            |

**Results and Discussion**

**Variation of rice gain color among 90 accessions**

From database of Plant Resources Center, Vietnam national gene bank (http://en.prc.org.vn/), all of 90 rice gain color accessions were selected with description based on Biodiversity International standard (Table 3). Of those the dominant number of accessions was variable purple and the smallest number (5 accessions) was purple color. Thus, this rice gain color population reflected local-colored rice landraces in North central of Vietnam.

| No | Seed coat color | Number of accession | Percentage (%) |
|----|-----------------|---------------------|----------------|
| 1  | Brown           | 13                  | 14.4           |
| 2  | Red             | 30                  | 33.3           |
| 3  | Variable purple | 42                  | 46.7           |
| 4  | Purple          | 5                   | 5.6            |

**DNA extraction**

The DNA concentration ranged from 150-300 ng/µl as shown in Fig. 1. A total DNA of 90 colored rice landraces were extracted following to the method of Zheng et al. [11]. PCR products were electrophoresed in the 1% agarose gel with 50ng/µl of Lamda DNA; and DNA concentration was measured by the spectrum nanodrop machine at the wavelength OD260/OD280.
The DNA products were shown to be high yield, good quality and concentration at 150-300 ng/µl which are good enough to implement further PCR assay (Fig. 1).

**Figure 1.** Total DNA in 1% agarose gel

**Analysis of genetic diversity of colored rice landraces using SSR markers**

The results of the PCR products were yielded DNA bands ranging 80-425 bp. At each locus, the size of alleles varied from 7 bp (RM 144) to 125bp (RM20590) (Fig. 2).

![Figure 2. PCR products of colored rice landraces amplified by using the RM144 primer](image)
In total, 184 alleles were scored at 40 loci, the number of allele per locus ranged from 2 to 12, and average of 5.7 alleles per locus. The number of polymorphic alleles per locus were from 3 (RM5364) to 12 (RM5647), and average of 7.1 alleles per locus. There were 5 marker pairs recorded for 4 alleles (RM27274, RM201, RM5948, RM347, RM138); 6 primer pairs recorded for 5 alleles (RM346, RM208, RM17231, RM3866, RM280, RM14226); 4 marker pairs recorded for 10 alleles (RM541 RM6314, RM23251, RM339) and only 2 marker pairs RM250, RM228 recorded for 11 alleles (Fig. 3).

Table 4 showed that 11 markers were found giving unique allele, including RM250, RM302, RM10926, RM208, RM227, RM17231, RM23251, RM5647, RM1376, RM339 and RM228; these unique alleles can be used to identify the colored rice landraces collection. For example, Khau cam xang variety (GBVN018073), originated from Con Cuong, Nghe An, can be identified using 3 markers RM10926, RM208 and RM5647. Similar to Plemamu landrace (GBVN014419) also recognized by using markers RM227, RM23251 and RM339; and Khau phach variety (GBVN008251), originated from Moc Chau, Son La, can be also identified by using two markers RM5647 and RM228.

**Table 4.** Polymorphic data using SSR markers for local colored rice landraces

| No | Locus | Chr. | No. observed alleles | Minimum allele size (bp) | Maximum allele size (bp) | No. unique allele | Acc. Number of landraces having unique allele | PIC value |
|----|-------|------|----------------------|--------------------------|--------------------------|-------------------|---------------------------------------------|-----------|
| 1  | RM250 | 2    | 11                   | 140                      | 187                      | 1                 | GBVN00201 3 (140bp)                         | 0.84      |
| 2  | RM302 | 1    | 8                    | 125                      | 162                      | 1                 | GBVN00178 61 (125bp)                        | 0.72      |
| 3  | RM10926 | 1    | 9                    | 140                      | 160                      | 2                 | GBVN00823 1 (140bp) GBVN018073 3 (160bp)    | 0.78      |
| 4  | RM541 | 6    | 10                   | 148                      | 195                      | -                 | -                                           | 0.80      |
| 5  | RM21969 | 7    | 9                    | 88                       | 102                      | -                 | -                                           | 0.85      |
| 6  | RM346 | 7    | 5                    | 140                      | 160                      | -                 | -                                           | 0.68      |
| 7  | RM27274 | 11   | 4                    | 220                      | 240                      | -                 | -                                           | 0.61      |
| 8  | RM201 | 9    | 4                    | 130                      | 158                      | -                 | -                                           | 0.60      |
| 9  | RM208 | 2    | 5                    | 160                      | 173                      | 1                 | GBVN018073 3 (173bp)                        | 0.41      |
| 10 | RM227 | 3    | 6                    | 95                       | 110                      | 1                 | GBVN014419 (110bp)                          | 0.49      |
| RM       | 38 | 9  | 140 | 165 | -  | -  | 0.84 |
|----------|----|----|-----|-----|----|----|------|
| RM 17231| 4  | 5  | 80  | 95  | 1  | GBVN00728 2 (80bp) | 0.73 |
| RM 27027| 11 | 8  | 165 | 182 | -  | -  | 0.81 |
| RM 20589| 6  | 6  | 242 | 270 | -  | -  | 0.73 |
| RM 144  | 11 | 6  | 220 | 275 | -  | -  | 0.76 |
| RM 23251| 8  | 10 | 210 | 267 | 1  | GBVN00419 9 (267bp) | 0.83 |
| RM 1233 | 11 | 9  | 150 | 190 | -  | -  | 0.76 |
| RM 5647 | 8  | 12 | 98  | 138 | 2  | GBVN00825 1(98bp), GBVN01807 3 (102bp) | 0.89 |
| RM 1376 | 8  | 7  | 180 | 205 | 1  | GBVN00944 6 (205bp) | 0.76 |
| RM 339  | 8  | 10 | 135 | 190 | 2  | GBVN00501 7(138bp), GBVN01441 9 (135bp) | 0.79 |
| RM 228  | 10 | 11 | 102 | 122 | 1  | GBVN00825 1 (118bp) | 0.82 |
| RM 5948 | 5  | 4  | 138 | 145 | -  | -  | 0.49 |
| RM 20590| 6  | 8  | 300 | 425 | -  | -  | 0.83 |
| RM 316  | 9  | 6  | 192 | 240 | -  | -  | 0.78 |
| RM 5473 | 4  | 8  | 87  | 112 | -  | -  | 0.84 |
| RM 224  | 11 | 7  | 120 | 157 | -  | -  | 0.68 |
| RM 3866 | 4  | 5  | 150 | 170 | -  | -  | 0.72 |
| RM 247  | 12 | 6  | 125 | 180 | -  | -  | 0.7  |
| RM6836  | 6  | 7  | 225 | 275 | -  | -  | 0.81 |
| RM347   | 3  | 4  | 207 | 240 | -  | -  | 0.48 |
| RM 270  | 12 | 5  | 110 | 140 | -  | -  | 0.77 |
| RM 1359 | 4  | 7  | 150 | 180 | -  | -  | 0.82 |
| RM 21077| 7  | 6  | 160 | 187 | -  | -  | 0.82 |
| RM 138  | 2  | 4  | 200 | 233 | -  | -  | 0.75 |
| RM 14226| 2  | 5  | 280 | 300 | -  | -  | 0.76 |
| RM 5536 | 1  | 8  | 140 | 165 | -  | -  | 0.85 |
| RM 5364 | 12 | 3  | 150 | 160 | -  | -  | 0.66 |
| Minimum  | 3  | 80 | 95  | 1   |    |    | 0.41 |
| Average  | 7.1|    |     |     |    |    | 0.74 |
| Maximum  | 12 | 300| 425 | 2   |    |    | 0.89 |
| Total    | 284|    |     |     |    |    |    |

After examining the SSR loci, the PIC values ranged from 0.41 (RM208) to 0.89 (RM5647), the average PIC value was 0.74. This result was lower than PIC value reported by Gowda et al. [7] where an average PIC was 0.84 in the analysis of the 45 Indian cultivated landraces. But this study's PIC value was higher than the studies of Freeg et al. [15] with an average PIC of 0.52 or higher than the results published by Soe et al. [16] with an average number of alleles of 6.83 and the average PIC reached 0.55.
Analysis of genetic relationships among the local-colored rice landraces.

Cluster analysis showed significant genetic variation among the landrace rice varieties studied, with genetic distance ranging from 0.76 to 0.93 (Fig. 4). With a genetic distance of 0.78, the cluster revealed 5 major groups.

Group I consisted of 59 landraces, in which the majority of collection’s seed coat color as variable purple (33 out of 59) and the Khau tang san cha (GBVN012565) and Naple la (GBVN003562) landraces were separated from the others; at the genetic correlation level of ~0.73, the rest of group I was divided into three subgroups:

Sub-group I-a consisted of 7 landraces: GBVN001912, GBVN002013, GBVN002019, GBVN002020, GBVN002056, GBVN002102, GBVN002509, in which 2 rice landraces as GBVN001912 and GBVN002013 having the highest similarity coefficient 0.88.

Sub-group I-b had 50 landraces, divided into 2 subgroups as I-b.1 and I-b.2. The sub-group I-b.1 consisted of 15 landraces in which 8 accessions collected from Northwest (GBVN002515, GBVN003926, GBVN003929, GBVN003969, GBVN004019, GBVN004020, GBVN004199, GBVN01454), 02 accessions from Northeast (GBVN004082, GBVN014654) and 5 accessions from North Central (GBVN005017, GBVN005034, GBVN005101,GBVN005102, GBVN005175). The similarity coefficient among these landraces ranged from 0.81 to 0.90

Sub-group I-b.2 consisted of 35 landraces with similarity coefficient ranging from 0.84 to 0.93. The pair of landraces (GBVN008674) and Khau ho he (GBVN008678) had the greatest similarity coefficient of 0.93 and interestingly, these two samples originated from North Central. Sub-group I-c had 2 landraces GBVN012565 and GBVN003562 with similarity coefficient of 0.83, both of them having red seed coat.
Figure 4. Dendrogram of genetic relationship among 90 colored rice genotypes based on 40 SSR markers

Group II had 7 landraces GBVN014419, GBVN014482, GBVN014471, GBVN014840, GBVN014846 and GBVN014850 obtained from Northwest and only GBVN017861 collected from North Central. The similarity coefficient of this group was from 0.80 to 0.88.

Group III: consisted of 4 landraces GBVN007715, GBVN008212, GBVN008231 and GBVN008251 with similarity coefficient ranging from 0.80 to 0.92. In this group, most of the genotypes originated from Son La province, only the Khau doi dang 2 (GBVN007715) originated from Thanh Hoa province.

Group IV: 18 landraces and divided into three sub-groups:

Group IV-a: comprising of 7 landraces GBVN002024, GBVN002093, GBVN002468, GBVN004003, GBVN003918, GBVN012593 and GBVN003921; all of these were collected from Northwest, only accession GBVN004003 had seed coat brown and the remaining were red seed coat. The similarity coefficient among this group ranged from 0.82 to 0.87.

Subgroup IV-b: contained 03 landraces: GBVN004153, GBVN013001, GBVN004688 with brown seed coat and only GBVN013005 was red seed coat. The similarity coefficient among this group ranged from 0.81 to 0.87.
Sub-group IV-c: consisted of 6 genotypes with red seed coat (GBVN013010, GBVN014220, GBVN007146, GBVN007151 and GBVN007282) and similarity coefficient of 0.83 to 0.92. Regarding to origin, this sub-group had 4 varieties collected from Thanh Hoa province (Central of Vietnam) and 2 varieties obtained from Son La province (Northwest).

Group V had two landraces Khau cam xang (GBVN018073 and GBVN005078) derived from North Central of Vietnam with the similarity coefficient of 0.83.

Conclusions

The genetic diversity of 90 local-colored rice accessions in Vietnam were evaluated using 40 SSR markers which were distributed through the 12 rice chromosomes. The results indicated that the numbers of polymorphic alleles ranged from 3 to 12 alleles per locus and average of 7.1 alleles per locus; 11 markers such as RM250, RM302, RM10926, RM208, RM227, RM17231, RM23251, RM5647, RM1376, RM339 and RM228 gave the unique allele for 10 colored rice landraces. Polymorphic Information Content (PIC) at analyzed locus was high with an average of 0.74. The similarity coefficients of the 90 landraces studied ranged from 0.76 to 0.93; at a level of 0.78; 90 colored rice landraces were divided into five groups. The majority of varieties with variable purple were in group I, accounting for 78.9%; interestingly, cluster IV and V consisted of only varieties with brown and red seed coat color. The results of this study has found 11 markers that can be used effectively for genetic diversity of colored rice and also provide useful materials and information for genetic resources conservation as well as breeding of colored rice in Vietnam.

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