RESEARCH ARTICLE

SSR based genetic diversity of pigmented and aromatic rice (*Oryza sativa* L.) genotypes of the western Himalayan region of India

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Abstract A set of 24 of SSR markers were used to estimate the genetic diversity in 16 rice genotypes found in Western Himalayas of Kashmir and Himachal Pradesh, India. The level of polymorphism among the genotypes of rice was evaluated from the number of alleles and PIC value for each of the 24 SSR loci. A total of 68 alleles were detected across the 16 genotypes through the use of these 24 SSR markers. The number of alleles per locus generated varied from 2 (RM 338, RM 452, RM 171) to 6 (RM 585, RM 249, RM 481, RM 162). The PIC values varied from 0.36 (RM 1) to 0.86 (RM 249) with an average of 0.62 per locus. Based on information generated, the genotypes got separated in six different clusters. Cluster 1 comprised of 4 genotypes viz; Zag 1, Zag 13, Pusa sugandh 3, and Zag 14, separated from each other at a similarity value of 0.40. Cluster second comprised of 3 landraces viz; Zag 2, Zag 4 and Zag10 separated from each other at a similarity value of 0.45. Cluster third comprised of 3 genotypes viz; Grey rice, Mushk budji and Kamad separated from each other at a similarity value of 0.46. Cluster fourth had 2 landraces viz; Kawa breed and Loual anzul, and was not sub clustered. Fifth cluster had 3 genotypes viz; Zag 12, Purple rice and Jhelum separated from each other at a similarity value of 0.28. Cluster 6 comprised of a single popular variety i.e. Shalimar rice 1 with independent lineage.

Keywords *Oryza sativa* L. · Genetic diversity · SSR markers · Polymorphism · Microsatellites

Introduction

Rice is one of the world's most important cereal crops. It belongs to grass family Poaceae ($2n = 12$). There are 25 species of genus *Oryza*, and only two species, namely *Oryza sativa* and *Oryza glaberrima* are cultivated (Onwueme and Sinha 1991). The crop constitutes the staple food of more than 65 % of the total population in India, 43 % of the total food grain production and 47 % of the total cereal production (Anonymous 2014). Knowledge of the genetic diversity of germplasm collections is an important foundation for crop improvement (Husaini et al. 2013). For thousands of years conventional breeding approaches have been used to improve rice cultivars, but there are difficulties in making genotype selections. Exploring diversity in a landrace-collection is very important for identifying new genes and improvement of the existing high yielding varieties (Brondani et al. 2006, Thomson et al. 2007). Landraces of rice are a significant genetic resource for rice improvement (Tang et al. 2002). Traditionally these are important reservoirs of valuable traits viz. taste, aroma, nutrition, medicinal, properties and other special uses. In order to maintain this crop diversity and use it in breeding programmes, collection, characterization and conservation of traditional landraces is of vital importance (Prabakaran et al. 2010, Husaini et al. 2013).

Western Himalayas are a repository of high altitude cold tolerant rice genotypes, which are classified into group VI (Glaszmann 1987). After the advent of Green revolution in India in late 1960s high yielding rice varieties were popularized in the traditional rice growing regions of Indian

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sub-continent. Owing to their better fertilizer response, high yield and more economic returns farmers adopted these varieties, and this led to loss of about three quarters of original varieties from the farm field. This genetic erosion of many valuable ‘specialty’ rice genotypes in the region went unnoticed. However, some inaccessible far flung areas remained untouched by this wave. These pockets of traditional rice landraces, particularly in high altitude marginal areas, are not fit for high yielding varieties of rice. In view of the growing significance of rare germplasm as gene pool resource, it has become imperative to document, conserve and characterize this rare germplasm (Husaini et al. 2013). This is the first study of its kind on these very important ‘specialty’ rice landraces, albeit neglected, of the Western Himalayan region.

Molecular markers are important for the characterization of germplasm and offer opportunities to delineate the genetic differences at the molecular level among the available diversity. Further, there is a possibility of redundant or duplicate accessions amongst these closely related genotypes. Marker based diversity analysis can therefore not only help in accurate identification and classification of the genotypes, but also in detection of any duplication. A range of molecular approaches are used to study the diversity in conserved germplasm. PCR based markers like simple sequence repeats (SSRs) and Amplified Fragment Length Polymorphism (AFLP) are widely used. SSRs are subset of tandemly repeated DNA families represented by extremely short nucleotide sequence repeats of 1–5 base pairs that are abundantly present in eukaryote genome, and provide co-dominant markers with Mendelian inheritance (Beckmann and Soller 1990). Simple sequence repeat (SSR) markers are reliable, reproducible, highly polymorphic and have high resolution (Ni et al. 2002). These markers are cost effective and can be carried out on high throughput (Coburn et al. 2003). The purpose of the present study was to develop DNA fingerprints of 16 pigmented and aromatic genotypes, mostly of Western Himalayan region (32° 44′–35° 2′ N and 74° 28′–75° 48′ E at altitude range of 1540–2200 MAMSL in Kashmir; and 31° 17′ N and 76° 51′ E at an altitude of 1,190 MAMSL in Himachal Pradesh (India) and evaluate for genetic diversity using SSR markers.

Materials and methods

Experimental material

The present study was carried out in the Centre for Plant Biotechnology, SKUAST-Kashmir, Shalimar Campus, Jammu and Kashmir, India. The plant material (leaves) was procured from Mountain Research Centre for Field Crops

(MRCFC), Khudwani, Anantnag. The list of selected genotypes along with brief description is given in Table 1.

DNA extraction

Young leaves at 5 leaf stage were harvested and placed in glassine bags, transported in ice and stored at –20 °C. Plant DNA was isolated using CTAB method as modified by Saghai-Maroofo et al. (1994). The extracted DNA was diluted in 800 µl TE (Tris EDTA buffer–10 mM Tris HCl, 1 mM EDTA, pH 8.0). The tubes were left for few hours at room temperature to allow DNA to dissolve. Quality and quantity of DNA was determined by UV visible spectrophotometer (HITACHI U- 2800 UV–VIS spectrophotometer).

PCR analysis and gel electrophoresis

A set of twenty-four SSR primers were used and the sequence of primers was obtained from website <http://www.gramene.org/microsat.txt> (Table 2). The PCR reaction was carried out using *Taq* polymerase in 20 µl reaction with 1X PCR buffer, 1.5 mM MgCl₂, 100 µM dNTPs, 0.25 µM Primer Forward, 0.25 µM Primer Reverse, 1U *Taq* Polymerase, and 75 ng DNA template. Thermal cycle program was set as follows: Initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation step at 94 °C for 1 min, annealing step at 55–60 °C for 1:30 min, elongation step at 72 °C for 2 min and final extension at 72 °C for 15 min (McCouch et al. 2002; Chakravarthi and Naravaneni, 2006). The SSR-PCR products were analyzed on 2.5 % agarose gel using DNA ladder (100 bp) and were visualized by staining with ethidium bromide.

Data analysis

The amplified products from SSR marker analysis were scored quantitatively for presence (1) or absence (0) for each marker allele-genotype combination. The similarity matrix was thus generated and dendrogram was constructed using UPGMA (Unweighted Pair Group Method using Arithmetic Averages) available in NTSYSpc 2.21p (Rohlf 2000). Similarity was estimated using SIMQUAL function of NTSYS, Polymorphism information content (PIC) value of a marker was calculated according to the following formula of Anderson et al. (1993).

$$PIC = 1 - \sum_{j=1}^n p_{ij}^2$$

Where, p_{ij} is the frequency of j th allele for the i th marker, and summed over on alleles PIC value exceeding 0.5 indicated presence of polymorphism for the alleles.

Table 1 List of the rice genotypes used in the present study

S. no	Genotype	Description
1.	Zag 1	Japonica type short and bold red rice; grown in Kupwara region at 1850–2200 MAMSL
2.	Zag 2	Japonica type short and bold red rice; landrace grown in Uri at 1850–2200 MAMSL
3.	Kund Zag (Zag 4)	Japonica type short and bold red rice; grown at 1850–2200 MAMSL
4.	Khuch (Zag 10)	Japonica type short and bold red rice; landrace grown in Larnoo at 1850–2200 MAMSL
5.	Cheolweon (Zag 13)	Japonica type short and bold red rice; introduction from Korea and grown at 1650 MAMSL at Khudwani
6.	Zag 14	Japonica type short and bold red rice; grown at 1850–2200 MAMSL
7.	Zager	Japonica type short offwhite; grown at 1850–2200 MAMSL
8.	Pusa sugandh-3	Scented Basmati type grown at 1540–1600 MAMSL; introduced variety grown in Budgam, Anantnag and Kulgam areas
9.	Grey rice	Long and medium bold grown at 1600–1650 MAMSL (introduced from Turkey)
10.	Purple rice	Long and medium bold purple colour grain; grown at 1600–1650 MAMSL (introduced but origin unknown)
11.	Kamad	Japonica type scented short and bold off-white; landrace grown at 1850–2200 MAMSL
12.	Kawa breed	Japonica type short offwhite; grown at 1850–2200 MAMSL
13.	Loual anzul	Japonica type short offwhite; grown at 1850–2200 MAMSL
14.	Jhelum	Indica type medium bold grain; variety released in 1996 and grown at 1540–1650 MAMSL
15.	Mushk budji	Japonica type scented short and bold off-white; landrace grown in Sagam, Larnoo etc. at 1850–2200 MAMSL
16.	Shalimar rice-1	Indica type medium bold grain; variety released in 1996 and grown in Valley at 1540–1600 MAMSL

Note: Zag 1–14 are accessions of red rice types maintained in germplasm bank at MRCFC, Khudwani

Pairwise comparison of the genotypes, based on the proportion of alleles, was used to measure the genetic similarity. Estimates of genetic similarity (measuring genetic distance) were calculated between all pairs of the genotypes according to Nei and Li (1979). The similarity coefficients (reverse genetic distance) were used to construct a dendrogram. Based on the analysis of pooled SSR marker data, the genotypes were classified into different clusters to assess the genetic diversity at the molecular level.

Results and discussion

The landraces studied in the present investigation have been cultivated since time immemorial in the mid-hills of Pir Panjal range of Western Himalayas in Kashmir, India (32° 44'S–35° 2'N and 74° 28'E–75° 48'E; 1540–2200 MAMSL). The ecotypes should not be genetically dissimilar as these are grown within narrow latitude range. However, even a single mutation is sometimes sufficient to cause appreciable difference in phenotype. For example, the aromatic short bold japonica rice landrace of the region, namely 'Mushk budji' is probably the result of a single deletion mutation in *badh-2*, leading to the production of fragrance (preliminary results in another study).

Number of alleles

Among 24 SSR primers used to generate marker profiles, eighteen SSR primers succeeded in amplifying the 16 genotypes, producing 68 alleles spread over 12 chromosomes (Fig. 1). The majority of the SSR loci differed appreciably in the number of alleles. The number of alleles produced from different primers ranged from 2 to 6, with an average of 3.77 alleles per primer (Table 2). In a genetic diversity analysis of *Oryza rufipogon* populations using 23 SSR primer pairs a total of 115 alleles were scored and the allelic number had ranged from 2 (RM 19) to 8 (RM 44) (Song et al. 2003). A maximum of 6 alleles each were scored by primers RM 249, RM 585, RM 481, RM 162. Six SSR primers namely RM 489, RM 252, RM 335, RM 161, RM 72, RM 222 showed no amplification despite varying annealing temp in gradient PCR from 55 to 60 °C. Significant correlation existed between the number of alleles at SSR loci and the PIC values.

Polymorphism of SSR markers

Polymorphic information content (PIC) value is reflection of allelic diversity and frequency among the genotypes. The PIC values varied from 0.36 (RM 1) on the chromosome 1–0.86 (RM 249) on the chromosome 5, while the

Table 2 Allele variation and polymorphic information content (PIC) for SSR loci across 16 selected rice genotypes

SSR marker	Primer sequence	Chromosome location no.	Annealing temp (°C)	No. of alleles	PIC value
RM 1	F gcgaaaacacaatgcaaaaa	1	55	3	0.36
	R gcgttggttggaacctgac				
RM 259	F tggagtttgagaggagg	1	56	5	0.72
	R ctgttgcatggtgccatgt				
RM 27	F ttctctctcaccacttca	2	55	3	0.54
	R tctttgacaagaggaaagaggc				
RM 452	F ctgatcgagagcgtaagg	2	57	2	0.49
	R gggatcaaaccacgtttctg				
RM338	F cacaggagcaggagaagagc	3	58	2	0.47
	R ggcaaaccgatcactcagtc				
RM 249	F ggcgttaaaggtttgcatg	5	56	6	0.86
	R atgatccatgaagggtcagc				
RM 585	F cagtettgctccgtttgtg	6	56	6	0.81
	R ctgtgactgacttggtcatagg				
RM 162	F gccagcaaaaccaggatccgg	6	58	6	0.75
	R caaggtcttgtgcgcttgccgg				
RM 234	F acagtatccaaggccctgg	7	56	3	0.54
	R aacgtgagacaaggacggag				
RM 481	F tagctagccgattgaatggc	7	56	6	0.83
	R ctccacctcctagtgttc				
RM 281	F ccggcgataaaacaatgac	8	56	3	0.65
	R ctccacctcctagtgttg				
RM 107	F agatcgaagcatcgcgcccag	9	57	3	0.61
	R actgcgtcctctgggttccgg				
RM 316	F ctagttagcatacatggc	9	58	3	0.61
	R acgcttatatgttacgtcaac				
RM 171	F aacgcgagacacgtacttac	10	55	2	0.47
	R acgagatacgtacgccttg				
RM 206	F cgttccatcgatccgtatgg	11	58	3	0.57
	R cccatgcgtttaactattct				
RM 21	F acagtattccgtaggcacgg	11	56	4	0.69
	R gtcccatgaggggtgtagac				
RM 19	F caaaaacagacagatgac	12	56	3	0.42
	R ctcaagatggacccaaga				
RM 20	F atctgtccctgcaggtcat	12	58	5	0.77
	R gaaacagaggcacatttcattg				

average PIC was calculated as 0.62 per locus (Table 3). There was correlation between the PIC values and the number of alleles at SSR loci. Comparatively, moderate mean PIC value (0.62) could be due to: (1) limited number of genotypes that are very well acclimatized to the local environment; (2) less differences in the contribution of the marker DNA regions; (3) gene pool with narrow genetic base; (4) less mutation rate in the di/tri repeat (Giarrocco et al. 2007). The maximum PIC value of 0.86 in our study was recorded for SSR marker RM 249, which has shown a

PIC value of 0.41 earlier (Zhu et al. 2012). Similarly, RM 481 which has been previously reported to show a PIC value of 0.49 (Prabakaran et al. 2010), 0.57 (Juneja et al. 2002) was highly informative and its PIC value was 0.83. This indicated that these two primers (RM249 and RM481) are effective and useful in determining the genetic difference among the rice genotypes of the Western Himalayas and to study the phylogenetic relationships between them.

A previous study on 48 commercial rice cultivars of Zhejiang province (China) revealed that 18 of the 32 SSR

Fig. 1 Agarose gels stained with ethidium bromide showing genetic polymorphism among rice (*Oryza sativa* L.) genotypes using SSR primers codes for genotypes shown in figure—1 Zag 1; 2 Zag 2; 4

Zag 4; 10 Zag 10; 12 Zager; 13 Zag 13; 14 Zag 14; PS Pusa Sughand-3; PR Purple Rice; GR Grey Rice; MB Mushk Budji; KA Kamad; KK Kawa-Kreed; LA Loual-Anzul; J Jhelum; SR Shalimar rice-1

primer pairs were polymorphic and generated 42 distinct reproducible bands with an average of 2.33 per primer pair and PIC value ranged from 0.1 to 0.5 (Zhu et al. 2012). The PIC values of RM 1 (0.21), RM 206 (0.16), RM 249 (0.41) as reported in that study (Zhu et al. 2012) were found to be lower than PICs of RM 1 (0.36), RM 206 (0.57), RM 249 (0.86) in the present study, implying thereby that the same primers provide different information content depending on genotypes under study.

Perusal of literature pertaining to SSR primers used in the present study reveals that RM1 has been widely used by rice workers. PIC values of 0.47 (Martin et al. 2012), 0.50

(Chakravorty and Gosh 2013), 0.53 (Shah et al. 2013), 0.21 (Zhu et al. 2012), 0.64 (Wong et al. 2009), 0.36 (present study) have been reported for RM1. Similarly, RM 206 has also been used by many rice workers and have found the PIC values of 0.68 (Martin et al. 2012), 0.57 (Shah et al. 2013), 0.16 (Zhu et al. 2012), 0.53 (Wong et al. 2009), which are more or less similar to that recorded in present study (0.57).

Comparison of the PIC values reported by different workers indicates that the discriminatory power of each SSR marker varies with variation in the genotypes under study. RM 21 recorded a PIC value of 0.69 which is higher

Table 3 Total percentage of polymorphism detected in 16 rice genotypes by SSR markers

Primer name	Total no. of bands	Total no. of monomorphic bands	Total no. of polymorphic bands	Percentage of polymorphism
RM 1	11	7	4	36.6
RM 259	16	10	6	52
RM 27	16	10	6	14.2
RM 452	8	5	3	37.5
RM 338	16	16	0	0.0
RM 249	12	7	5	41.6
RM 585	16	6	10	62.5
RM 162	12	8	4	33.3
RM 234	12	7	5	46.45
RM 481	15	9	6	40
RM 281	12	10	2	15.38
RM 107	9	5	4	44.4
RM 316	8	5	3	37.5
RM 171	6	4	2	33
RM 206	9	3	6	66.6
RM 21	14	10	4	31.25
RM 19	13	10	3	20
RM 20	8	3	5	62

than that reported by ShuKun et al. 2010 (0.60), Chakravorty and Gosh 2013 (0.55). Similarly, RM 316 has shown a PIC value of 0.44 (ShuKun et al. 2010), 0.46 (Wong et al. 2009) while in the present study it was 0.61. RM 234 has shown a PIC value of 0.33 (Shah et al. 2013), and 0.45 (Juneja et al. 2002) but in the present study it was 0.54. Coincidentally, the PIC value of RM 19 was the same (0.42) as reported earlier (Zhu et al. 2012). These variations could be due to DNA sequence variations among the alleles at the sites of primer binding and due to the differences in DNA length between these primer binding sites. It is not at all necessary that all primers succeed in detecting these variations. In our study, we found 6 SSR markers that failed to amplify, and these were RM 489, RM 252, RM 335, RM 161, RM 72, RM 2.

Similarity coefficients between genotypes increasing genetic distance

A similarity coefficients matrix based on the proportion of shared SSR alleles was used to establish the level of relatedness between each pair of the cultivars/landraces. Pairwise genetic similarity coefficients varied from 0.00 to 0.647 (Table 4). Majority of the similarity coefficients were less than 0.40, which revealed that the genotypes selected in the present study possessed a very good spectrum of genetic diversity. Highest genetic similarity coefficient was found as 0.647 (between Zag 2 and Zag 4)

followed by 0.57 (between Grey rice and Mushk budji), while the least similarity coefficients (0.00) were between Zager and Kamad, Grey rice and Jehlum, Zag 10 and Jehlum, Purple Rice and Jehlum, Grey rice and Shalimar rice 1.

Cluster analysis and genetic divergence pattern

The input matrix for the genetic diversity/relatedness among the genotypes was prepared for SSR markers alleles at different loci leading to generation of similarity values among the genotypes studied. The genotypes got separated into six different clusters (Fig. 2). Cluster I consist of four genotypes (Zag 1, Zag 13, Pusa Sugandh 3, Zag 14) and is further divided into two sub-clusters (Ia and Ib) separated from each other at a similarity value of 0.40. It is quite striking that Pusa Sugandh 3 fits in this cluster, as unlike others in the group it is a long slender basmati type genotype. Cluster II consists of three genotypes (Zag 2, Zag 4, Zag 10) and is further divided into two sub-clusters IIa and IIb separated from each other at a similarity value of 0.45. This was expected as these genotypes possess similar seed characteristics and are distributed within a narrow altitudinal range. Cluster III consisted of three genotypes (Grey Rice, Mushk budji, Kamad) and was divided into two sub-clusters (IIIa and IIIb) separated from each other at a similarity value of 0.46. Mushk budji and Kamad are two famous aromatic landraces of Kashmir

Table 4 Similarity matrix for 16 rice genotypes based on SSR data

Genotypes	G1 (Zag-1)	G2 (Zag-2)	G3 (Zag-4)	G4 (Zag-10)	G5 (Zager)	G6 (Zag-13)	G7 (Zag-14)	G8 (Pusa Sugandh-3)	G9 (Purple Rice)	G10 (Grey Rice)	G11 (Mushk budji)	G12 (Kamad)	G13 (Kawa Kreed)	G14 (Loual Anzul)	G15 (Jhelum)	G16 (Shalimar rice-1)
G1 (Zag-1)	1															
G2 (Zag-2)	0.25	1														
G3 (Zag-4)	0.2273	0.6471	1													
G4 (Zag-10)	0.1739	0.4667	0.375	1												
G5 (Zager)	0.2273	0.1818	0.3	0.1429	1											
G6 (Zag-13)	0.4	0.2	0.2	0.1765	0.3846	1										
G7 (Zag-14)	0.2857	0.2222	0.2632	0.4118	0.1	0.2941	1									
G8 (Pusa Sugandh-3)	0.4167	0.2	0.2857	0.3846	0.3333	0.5556	0.5	1								
G9 (Purple Rice)	0.1818	0.087	0.0385	0.05	0.25	0.125	0.1	0.1333	1							
G10 (Grey Rice)	0.1053	0.2	0.25	0.3333	0.0588	0.1667	0.3333	0.4	0.2	1						
G11 (Mushk budji)	0.08	0.1818	0.2727	0.5	0.0909	0.1765	0.2632	0.2857	0.087	0.5714	1					
G12 (Kamad)	0.0357	0.1304	0.12	0.2381	0.0	0.2222	0.3	0.2	0.08	0.4667	0.5	1				
G13 (Kawa Kreed)	0.1053	0.2632	0.2632	0.1111	0.0476	0.2	0.3333	0.1429	0.0	0.2	0.0909	0.2632	1			
G14 (Loual Anzul)	0.1905	0.0909	0.0833	0.0476	0.1	0.3333	0.0909	0.125	0.0476	0.1111	0.1304	0.2381	0.4118	1		
G15 (Jhelum)	0.0952	0.1	0.1	0.0	0.2941	0.1765	0.0435	0.0	0.3333	0.0	0.0476	0.0909	0.1111	0.25	1	
G16 (Shalimar rice-1)	0.2143	0.1429	0.125	0.0909	0.1429	0.2	0.0769	0.2	0.1429	0.0	0.1429	0.0667	0.1667	0.0667	0.273	1

Fig. 2 Dendrogram depicting the genetic relationship among 16 genotypes of rice (*Oryza sativa* L.) based on SSR data using UPGMA

which have been registered as farmers' varieties and grow in high-altitude areas with cold climate (Husaini et al. 2009). Cluster IV consists of two genotypes (Kawa creed and Laul anzul) and was not sub-clustered. Cluster V consists of two sub-clusters Va and Vb separated from each other at a similarity value 0.28 and sub-cluster Va contained one genotype (Zager) and Vb contained two genotypes (Purple Rice and Jehlum). Cluster VI is independent and separated from cluster V at a similarity value 0.19 and consisted of only one genotype (Shalimar rice 1). It is a distinctly different genotype that was bred by crossing and released in the past decade as a blast tolerant variety for valley temperate regions (Parra et al. 2009).

Conclusion

Owing to limited geographical distribution, we had anticipated that most of the red rice types would show narrow genetic base and therefore fall within a single group, and some accessions would simply turn out to be duplicates. The above results, however, show considerable divergence between the genotypes which could be detected even by using limited (two dozen) SSR markers. This could possibly be due to characteristics of a peculiar hill ecology, where the size of each niche narrows down further due to steep and drastic differences in edaphic conditions, temperature, moisture, sunlight angle, wind direction etc.

caused due to variation in slope, altitude, shading etc. This observation has enormous implications for future studies on gene/allele mining, as germplasm in Western Himalayas could serve as a vital resource of genetic repository for marginal areas.

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