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Genetic Diversity Analysis of NERICA Lines and Parents Using SSR Markers

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LH and MMR designed the study, performed the statistical analysis and wrote the protocol. Author SI wrote the first draft of the manuscript. Authors MMR, MAI, SI and SR managed the analyses of the study. Authors MMR and SI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Diversity at molecular level among 10 genotypes was evaluated using Simple Sequence Repeat (SSR) markers. The primer RM510 is for the trait salt tolerant, RM351 for drought tolerant and RM215 for short duration. The 10 rice genotypes showed clear diversification for the primers RM510, RM351 and RM215. A total of 10 alleles were detected among the 10 rice genotypes. The number of allele locus^{-1} ranged from 3 (RM510 and RM215) to 4 (RM351) with an average of 3.33 allele locus^{-1}. On average, 40% of the 10 rice genotypes shared a common major allele ranging

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from 30% (RM351) to 50% (RM510) common allele at each locus. The PIC value ranged from 0.492 (RM510) to 0.745 (RM351) with an average of 0.608 locus^{-1}. The highest PIC value (0.745) was obtained from RM351, followed by RM510 (0.492) and RM215 (0.586). However, RM510 (0.492) and RM215 (0.586) could be conceded as least powerful markers with lower PIC values. The 10 genotypes were grouped into 4 clusters based on UPGMA method. Using 27% similarity as the threshold for UPGMA clustering, we observed four major genetic clusters. Cluster I, II, III and IV contained 1, 3, 1 and 5 genotypes respectively. These results revealed that markers RM351 would be best in screening 10 rice genotypes followed by RM510 and RM215. The information about the genetic diversity will be very useful for proper identification and selection of appropriate parents for future breeding programs.

Keywords: Genotype; primer; molecular; diversity; SSR.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for over 60% of the world's population [1]. In the coming 30 years, the world will require 70% more rice than it requires today. In respect of area and production of rice, Bangladesh ranks fourth following China, India and Indonesia [2]. Rice plays an important role in the Agro-economy and national health of Bangladesh. Bangladesh earns about 21.10% of her gross domestic product (GDP) from agriculture [3]. It provides about 75% of the calorie and 66% of the protein in the average daily diet of the people of Bangladesh [4]. In Bangladesh, rice is grown in three seasons (Aus, Aman and Boro) of the year. In three distinct seasons namely Aus, Aman and Boro reported with the production of 2.63, 12.74 and 18.53 million tons, respectively [5]. However, both area (1.41 million ha) and yield (2.05 thac^{-1}) of Aus rice were very low compared with other two seasons [6]. The total cropped area of Bangladesh is 33422 thousand acres of which 26130 thousand acres are used for rice cultivation and the production of rice is 28931 thousand metric tons [7]. NERICA has been introduced recently in Bangladesh for growing in drought prone areas. The term NERICA stands for New Rice for Africa, an extended family of some 3000 siblings. NERICA is the product of interspecific hybridization between the cultivated rice species of Africa (*O. glaberrima*) and Asia (*Oryza sativa*). Development of improved rice varieties, with stress tolerance traits from NERICA could significantly increase productivity [8]. Drought is the most devastating among abiotic stresses and it depresses yield by 15-50% depending on the vigour and period of stress in rice [9]. Severe drought during flowering and ripening stages reduces the grain yield by up to 70%. Drought affects rice crops in three different cropping seasons namely pre-kharif, Kharif, and Rabi seasons, where rice is the main staple food in Bangladesh accounting for more than 80% of the total cultivable land of the country. Developing high yielding and drought resistant varieties for rainfed area is a priority for improving rainfed rice production. Information about genetic diversity facilitates the selection of parental genotypes from random population. Molecular markers provide information that helps to define the distinctiveness of germplasms and their ranking according to the number of a close relative and their phylogenetic position. Several molecular markers viz. SSRs, RFLP [10], RAPD [11], ISSRs [12], AFLP [13] and SNPs [14] are presently available to assess the variability and diversity at molecular level [15]. With the development of a wide range of molecular techniques, marker-assisted breeding is now used to enhance traditional breeding programs to improve crops [16]. Among the several classes of available DNA markers, microsatellite or simple sequence repeat (SSR) markers are considered the most suitable due to their multi-allelic nature, high reproducibility, co-dominant inheritance, abundance and extensive genome coverage. Several studies have found that generic SSRs are useful for estimating genetic relationship and at the same time provide opportunities to examine functional diversity in relation to adaptive variation [17]. As a consequence, they are very informative markers that can be used for many population genetics studies, ranging from the individual level (e.g. clone and strain identification) to that of closely related species.

2. MATERIALS AND METHODS

2.1 Plant Materials

In this experiment, a total of ten NERICA lines (seven mutant lines along with three parents) of advanced generations were used

which were obtained from Bangladesh Institute of Nuclear Agriculture (Table 1).

Table 1. List of the genotypes used in the experiment

Sl. no	Genotypes	Source
1.	N_4/350/P-4(5)	Bangladesh Institute of Nuclear Agriculture (BINA)
2.	N_{10}/350/P-5-4	
3.	N_4/250/P-1(2)	
4.	N_1/250/P-6-2	
5.	N_4/250/P-2(6)-26	
6.	N_{10}/300/P-7-1	
7.	N_{10}/300/P-2(1)-4	
8.	N_1 Parent	
9.	N_4 Parent	
10.	N_{10} Parent	

Here, N_1= NERICA-1, N_4= NERICA-4, N_{10}= NERICA-10

2.2 Collection of Leaf Samples and DNA Extraction

Samples were collected from young, vigorous leaves from 21-day old seedlings to extract genomic DNA. At first, the healthy portion of the youngest leaves of the tiller was cut apart with sterilized scissors and washed in ethanol (70%) and distilled water. DNA was extracted from the leaves of each genotype using the Cetyl Trimethyl Ammonium Bromide (CTAB) mini-prep method.

2.3 Primer Selection and DNA Amplification

Primers were selected on the basis of band resolution intensity, the presence of smearing, consistency within individuals and potential for population discrimination. Primers which showed clear polymorphism were used for diversity analysis. The details of the primers are given in Table 2. The following PCR materials were used for PCR: 1 µl 10× PCR buffer, 0.6 µl $MgCl_2$, 0.2 µl Taq DNA polymerase, 1 µl forward primer, 1µl reverse primer, 1 µl dNTPs, 3.2 µl sterilized dH2O and 2µl template DNA for a total volume of 10 µl. For amplification, the thermal cycler was set at 1 cycle for 5 min at 94°C as an initial hot start and strand separation step. This was followed by 2nd program having 34 cycles, which comprises denaturation (94°C) for 1 min, annealing (55°C) for 1 min and primer elongation (72°C) for 2 min. Finally, 1 cycle of 7 min at 72°C was used for final extension and amplified products were stored at -20°C until further use. The amplification products were separated on 1.5% agarose gels in 0.5× TBE buffer. The DNA band patterns were visualized under UV light and photographed using a Polaroid camera.

2.4 Analysis of SSR Data

The summary statistics including the number of alleles per locus, major allele frequency, gene diversity and Polymorphic Information Content (PIC) values were determined using POWER MARKER version 3.23 [18], a genetic analysis software. Molecular weights for microsatellite products, in base-pairs, were estimated with Alpha Ease 4C software. The individual fragments were assigned as alleles of the appropriate microsatellite loci. Allele molecular weight data also used to determine the genetic distance for dendrogram reconstruction based on neighbor-joining method [19]. Bootstrapping of the UPGMA tree was performed using POWER MARKER with 1000 iterations followed by the PHYLIP CONSENSE module with the majority rule setting [20] a model based cluster analysis was then performed using the program where Pi is the allele frequency for the i-th allele [21]. The 10 lines were clustered based on the STRUCTURE version 2.1 [22]. Polymorphic Information Content (PIC) or Expected heterozygosity for each SSR marker was calculated based on the formula Hn= 1-Pi^2, matrix of genetic similarities using the

Table 2. Some details of the SSR markers used for polymorphism survey

Primer name	Sequence (5´- 3´)	Expected PCR product size	Annealing temp. (°C)
RM510	F. CGGATTAGTTTCTCGCC R. TGAGGACGACGAGCAGATTC	122	55
RM351	F. CCATCCTCCACCGCCTCTCG R. TGGAGGAAGGAAAGGGGACG	134	55
RM215	F. CAAAATGGAGCAGCAAGAGC R. TGAGCACCTCCTTCTCTGTAG	148	55

Unweighted Pair Group Method with arithmetic averages (UPGMA) the cluster analysis and dendrogram construction were performed with NTSYSPC (Version 2.1). Genetic distance values (D) [21] were calculated as:

$$D = -I_n J_{xy} / \Sigma J_x J_y$$

Where, $Jx=\Sigma Xi^2/r$ in population X, $Jy=\Sigma Yi^2/r$ in population Y, $Jxy=\Sigma XiYi$, Xi and Yi are the frequency of the i-th allele of a given locus.

3. RESULTS

In the present study, three SSR motifs were polymorphic and produced a varying number of alleles with different size ranges (Table 3). A total of 10 alleles were detected among the 10 rice genotypes. The number of allele locus^{-1} ranged from 3 to 4 with an average of 3.33 allele locus^{-1}. The locus RM351 had the highest number of alleles (4) and both RM510 and RM215 contained the lowest number of alleles (3). RM351 had high genetic diversity (0.780), while RM510 had the lowest genetic diversity (0.580) with a mean diversity of 0.673 (Table 4). The overall size of amplified products ranged from 107bp in locus RM510 to 174bp in locus RM215. On average, 40% of the 10 rice genotypes shared a common major allele ranging from 30% (RM351) to 50% (RM510) common allele at each locus (Table 4). The frequency of alleles at 3 SSR loci of 10 rice genotypes was shown in Table 4.Polymorphism information content (PIC) value is a reflection of allele diversity and frequency among the varieties. PIC value of each marker can be evaluated on the basis of its alleles. PIC varied significantly for all the studied SSR loci. In the present study, the level of polymorphism among the 10 rice genotypes was evaluated by calculating PIC values for each of the 3 SSR loci. The PIC values ranged from 0.492 (RM510) to 0.745 (RM351) with an average of 0.608 per locus (Table 3). The highest PIC value (0.745) was obtained from RM351, followed by RM510 (0.492) and RM215 (0.586). However, RM510 (0.492) and RM215 (0.586) could be conceded as the least powerful markers with lower PIC values. A dissimilarity matrix was used to determine the level of relatedness among the cultivars studied. The values of the pair-wise comparison of Nei's (1983) of genetic distance (D) between genotypes were computed from combined data for the 3 primers ranged from 0.00 to 1.00 (Table 6). The pair-wise genetic dissimilarity indices (Table 6) indicated that the highest genetic dissimilarity (100%) was observed among G4 with G1, G2 and G3; G6 with G1, G2 and G3; G7 with G1, G2 and G3; G8 with G1, G2 and G5; G9 with G1, G2, G3, G5 and G6; and G10 with G1, G2, G3 and G5. These pairs were followed by G5 with G1, G2, G3 and G4 (66.7%); G6 with G5 (66.7%); G7 with G4, G5 and G6 (66.7%); G8 with G3, G4 and G6 (66.7%); G9 with G7 and G8 (66.7%); G10 with G6 (66.7%). Both, G1 and G2, were found in duplicate (i.e., 0.00% dissimilarity that means 100% similarity). The lowest genetic dissimilarity (33.3%) among rice genotypes was between G1 and G3; G2 and G3; G4 and G6; G8 and G7; G4 and G10; G7 and G10; G8 and G10; G9 and G10. (Here, G1=N_4/350/P-4(5), G2=N_{10}/350/P-5-4, G3=N_4/250/P-1(2), G4=N_1/250/ P-6-2, G5=N_4/250/P-2(6)-26, G6=N_{10}/300/P-7-1, G7=N_{10}/300/P-2(1)-4, G8=N_1 Parent, G9=N_4 Parent and G10=N_{10} Parent). Cluster analysis was performed using the UPGMA method to group the studied genotypes based on similarity coefficient (Fig. 4). Using 27% similarity as the threshold for UPGMA clustering, we observed four major genetic clusters. Cluster I, II, III and IV contained 1, 3, 1 and 5 genotypes respectively. Among the total genotypes those which showed comparatively same banding patterns were grouped into the same cluster.

Table 3. Data of "summary statistics-l" of 10 rice genotypes for 3 SSR markers

Locus	Repeat motif	Chr. location	Allele size ranges (bp)	No. of allele	Rare allele	Null allele	PIC
RM510	(GA)15	6	107-125	3	0	0	0.492
RM351	(CCG)9(CGAAG)4	7	135-171	4	0	1	0.745
RM215	(CT)16	9	153-174	3	0	0	0.586
Mean	**---------**	**---------**	**---------**	**3.333**	**0**	**0.333**	**0.608**

*Here, PIC= Polymorphic Information Content, *Rare alleles are defined as alleles with a frequency less than 0.05 (5%)*

Table 4. Data of "summary statistics-II" of 10 rice genotypes for 3 SSR)markers

Locus	Sample size	Major allele		Availability	Gene diversity	Heterozygosity
		Size (bp)	Freq.			
RM510	10.0	107	0.500	1.00	0.580	0.000
RM351	10.0	145	0.300	1.00	0.780	0.000
RM215	10.0	174	0.400	1.00	0.660	0.000
Mean	**10.0**	**142**	**0.400**	**1.00**	**0.673**	**0.000**

**Major allele is defined as the allele with the highest frequency*

Table 5. List of genotypes in four clusters

Cluster number	Genotypes
I	G5
II	G1, G2, G3
III	G9
IV	G7, G8, G4, G10, G6

Here, G1=N_4/350/P-4(5), G2=N_{10}/350/P-5-4, G3=N_4/250/P-1(2), G4=N_1/250/P-6-2, G5=N_4/250/P-2(6)-26, G6=N_{10}/300/P-7-1, G7=N_{10}/300/P-2(1)-4, G8=N_1 Parent, G9=N_4 Parent and G10=N_{10} Parent

4. DISCUSSION

A total of 10 alleles were detected among the 10 rice genotypes. The number of alleles per locus ranged from 3 in RM510 and RM215 to 4 in RM351 with an average of 3.33 allele locus^{-1}.Furthermore, the average number of alleles per locus obtained in the present study was smaller than that reported in previous studies [23]. For example, Kuroda et al. [24] reported an average of 9.28 alleles per locus over 7 SSR loci and Rashid et al. [25] who recorded 5.167 alleles per locus using a small set of three SSR markers on 10 varieties.RM351 had high genetic diversity (0.780), while RM510 had the lowest genetic diversity (0.580) with a mean diversity of 0.673. It was observed that marker detecting the lower number of alleles showed a lower number gene genetic diversity than those detected a higher number of alleles which revealed high genetic diversity. The maximum number of repeats within the SSRs was also positively correlated with the genetic diversity. This result was consistent with previous work done by Herrera et al. [26], who observed that the gene diversity at each SSR locus was significantly correlated with the number of alleles detected, number of repeat motif and with the allele size range.Major allele is defined as the allele with the highest frequency and also known as the most common allele at each locus. The frequency of most common allele at each locus ranged from 30% to 50% with a mean frequency of 40%. Sajib et al. [27] also found that 56% of the 12 rice accessions shared a common major allele at any given locus ranging from 41% (RM163, RM590, and RM413) to 91% (RM510) common allele at each locus. Sajib et al. [27] also found a moderate level of diversity exists among 9 loci studied across 12 rice accessions, ranged from 0.15 to 0.75 with an average of 0.54. Polymorphism information content (PIC) value is a reflection of allele diversity and frequency among the varieties. PIC value of each marker can be evaluated on the basis of its alleles and it varied greatly for all the SSR loci tested. The PIC values ranged from 0.492 (RM510) to 0.745 (RM351) with an average of 0.608 per locus. According to the early reports on the PIC values ranged from a low of 0.24 to a high of 0.92 and averaged 0.61 [15], 0.19 to 0.90 with an average of 0.75 [28], which is markedly higher than the result in this study. Upadhyay et al. [29] also reported the average PIC value of 0.78 which is higher than the result of this study. These results revealed that markers RM351 would be best in analyzing the diversity of 10 rice genotypes followed by RM510 and RM215.The dissimilar matrix was used to determine the level of relatedness among the cultivars studied. Pair-wise estimates of dissimilarity ranged from 0.00 to 1.00 (Table 6). Generally, modern rice cultivars share a relatively narrow genetic background, when compared to the unexplored vast variability existing in rice landraces worldwide. Saini et al. [30] also reported almost similar values of similarity coefficient among 18 basmati and non-basmati varieties using molecular markers. Likewise, similarity coefficients ranging from 0.24 to 0.92 were observed in eight basmati accessions originating from Pakistan and one solitary indica accession for the SSR analysis [31]. Siwach et al. [32] also observed a higher level of similarity ranging from 0.67 to 0.91 among basmati and non-basmati long grains indica rice varieties using microsatellite markers. One of the reasons for this high level of similarity recorded by the present and previous studies could be due to intra-specific variation in the germplasms used. Genotypes having 100% similarity with each other were found as a duplicate. Sajib et al. [27] also found Deepa and Patnai-23 as duplicate. All of the 10 rice

genotypes were grouped in four main clusters. Pervaiz et al. [33] also showed four clusters comprised of his specimens. Tabkhkar et al. [32] also grouped 10 rice genotypes with SSR markers into four main clusters. Among the total genotypes those which showed comparatively same banding patterns were grouped into same cluster. In this study, the larger range of similarity values for cultivars revealed by microsatellite markers provides greater confidence for the assessments of genetic diversity and relationships, which can be used in future breeding programs. With the aid of microsatellite markers and clustering data, different distantly related rice genotypes may be combined by inter-crossing genotypes, for instance, one rice genotypes with other rice genotypes from different clusters to get hybrid varieties with highest heterosis.

Table 6. Summary of Nei's (1973) genetic distance values among 10 rice genotypes using 3 SSR markers

Genotype	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
G1	0.000									
G2	0.000	0.000								
G3	0.333	0.333	0.000							
G4	1.000	1.000	1.000	0.000						
G5	0.667	0.667	0.667	0.667	0.000					
G6	1.000	1.000	1.000	0.333	0.667	0.000				
G7	1.000	1.000	1.000	0.667	0.667	0.667	0.000			
G8	1.000	1.000	0.667	0.667	1.000	0.667	0.333	0.000		
G9	1.000	1.000	1.000	0.667	1.000	1.000	0.667	0.667	0.000	
G10	1.000	1.000	1.000	0.333	1.000	0.667	0.333	0.333	0.333	0.000

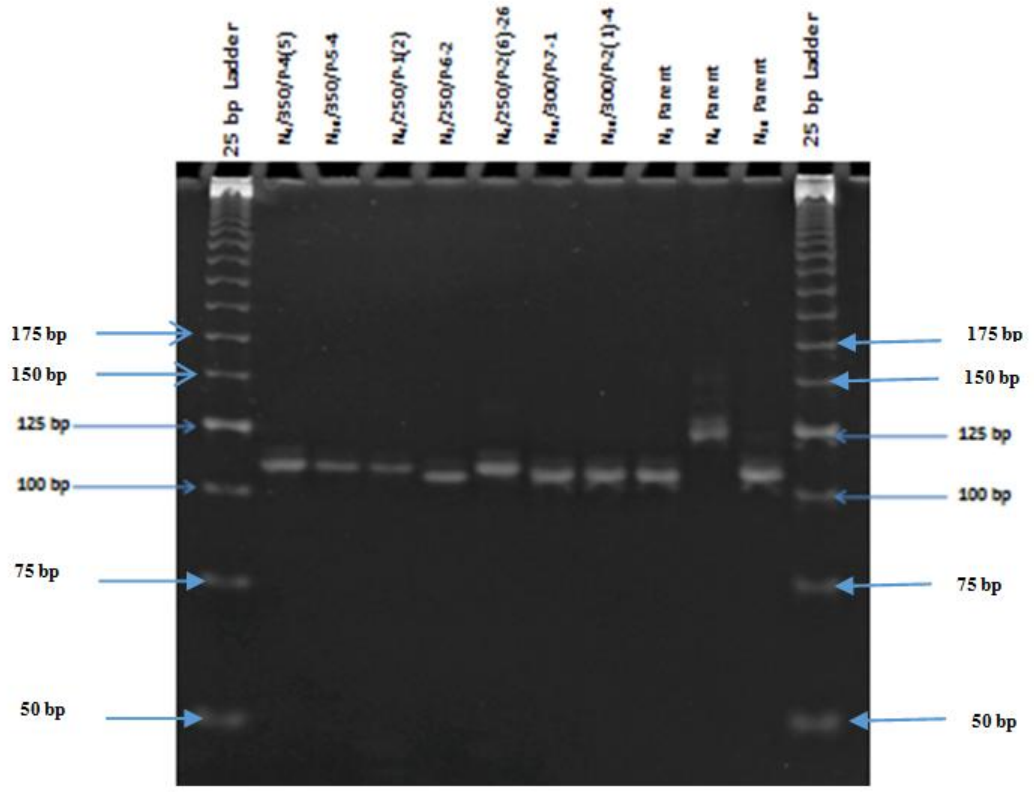

Fig. 1. SSR profiles of 10 NERICA lines using primer RM510

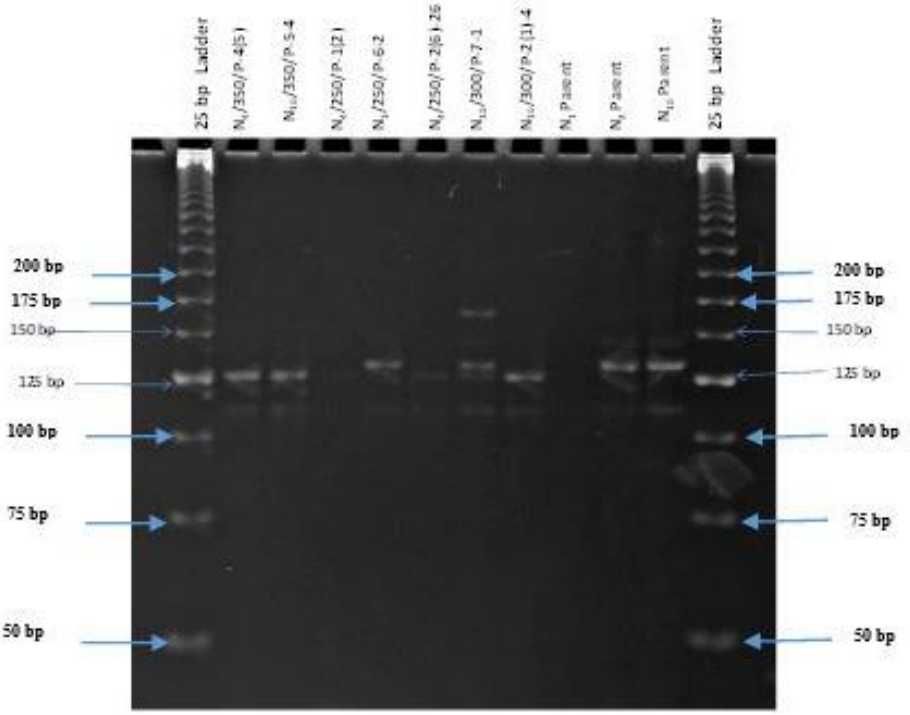

Fig. 2. SSR profiles of 10 NERICA lines using primer RM351

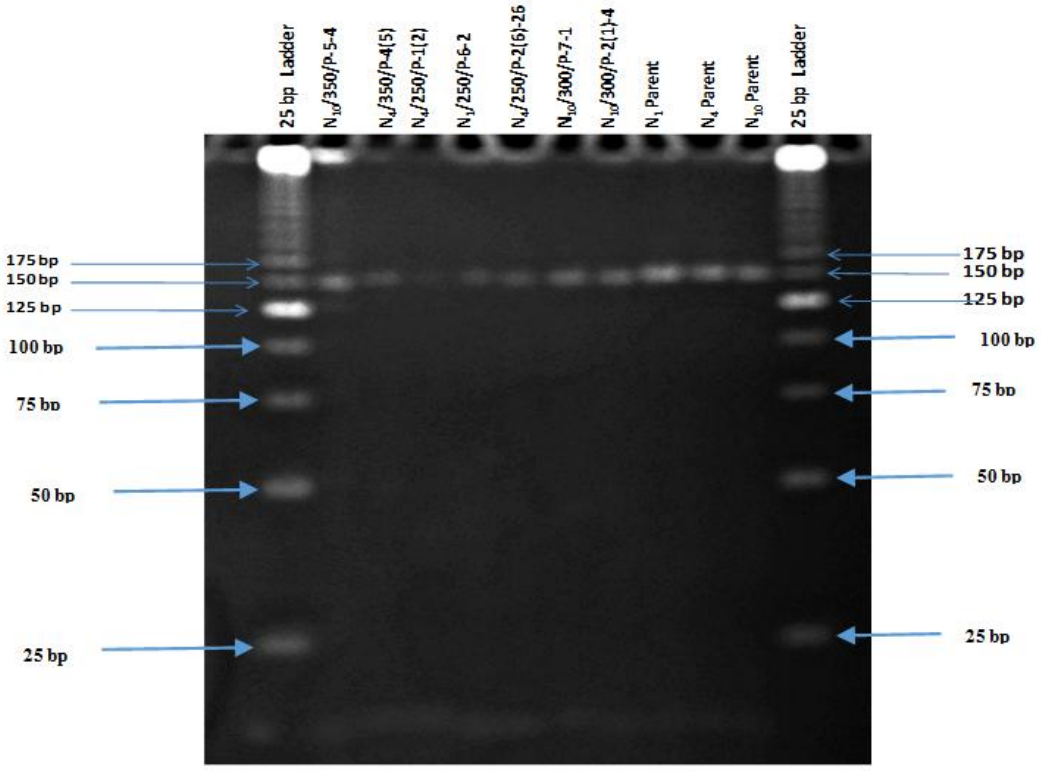

Fig. 3. SSR profiles of 10 NERICA lines using primer RM215

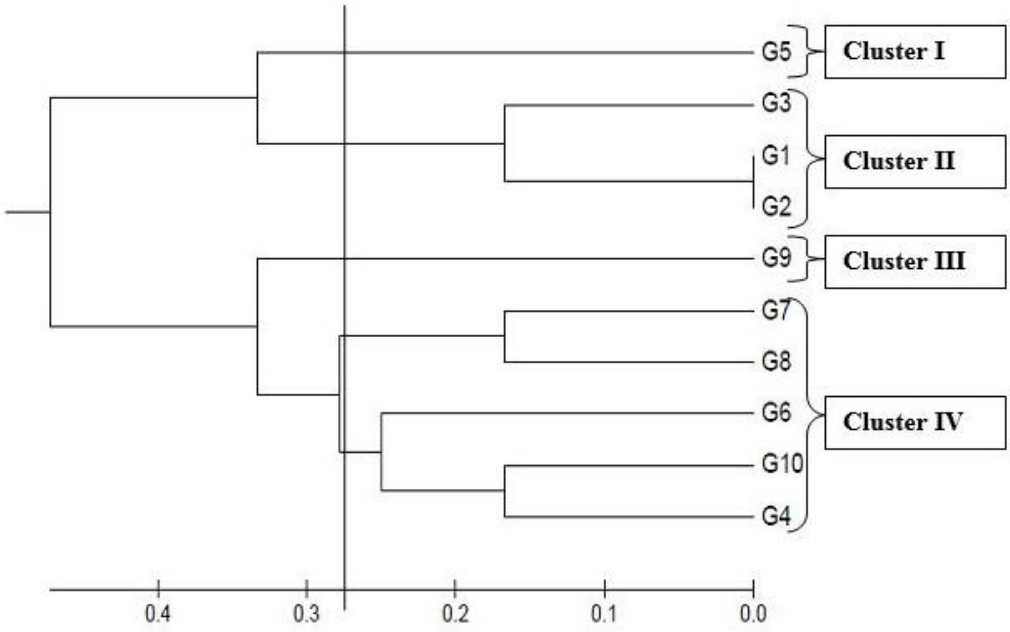

Fig. 4. UPGMA dendrogram based on Nei's (1973) genetic distance, summarizing data on differentiation among 10 rice genotypes according to SSR analysis

5. CONCLUSION

An SSR based screening of 10 rice genotypes using 3 SSR markers demonstrated a total of 10 alleles with an average of 3.33 alleles per locus. The highest PIC value (0.745) was obtained from RM351 followed by RM510 (0.492) and RM215 (0.586). However, RM510 (0.492) and RM215 (0.586) could be conceded as the least powerful markers with lower PIC values. Therefore, it can be concluded that RM351 would be best for screening 10 rice genotypes followed by RM510 and RM215.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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