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Research Note

Genetic diversity and DNA fingerprinting of rice varieties of Manipur using microsatellite markers

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Abstract
Rice variety differentiation was based on morphological descriptors (DUS) before the advent of genomic and proteomic technology. DNA fingerprinting is becoming an important molecular marker approach because of its wide application in varietal protection, classification, and conflict settlement. In the present study, genetic diversity and variability among the 11 rice varieties of Manipur at the molecular level was studied using 42 SSR markers. The genotypic data of the SSR markers revealed four sub-populations. Cluster analysis indicated the close similarity between RC Maniphou 12 and RC Maniphou 15. To create a DNA fingerprinting database for 11 rice varieties, three separate multiplex PCR systems using different combinations of 11 polymorphic SSR markers based on amplicon size were successfully developed . These were able to distinguish the distinctness of all the eleven released rice varieties.

Keywords: DNA fingerprinting, SSR, multiplex PCR, Rice, Microsatellite

Rice (*Oryza sativa* L.) is the most widely known and dominant food crop. About half of the world's population are dependent upon rice as a staple food (Ngangkham *et al*., 2018; Sellamuthu *et al*., 2011). The crop is cultivated abundantly in a wide range of geographical areas and agro-climatic conditions (Ngangkham *et al*., 2019). It is reported that more than 100 countries grow rice as a major crop with 90% of the total global production from Asian countries (Papademetriou, 2000). The origin of rice was reported near the North-Eastern part of India and southern China, both of which fall into the Himalayan subtropical upland region (Zhao *et al*., 2010; Choi *et al*.,2017). Additionally, India is the largest producer of rice. However, the ever-increasing global population's food demand in terms of quantity and quality with the change of lifestyle and change in climatic conditions lead to the transition of cropping pattern, thus influencing the crop's adaptation. According to FAO, the productivity of the rice grain is required to be enhanced by up to 50% more than the present production by the year 2050 (Khush, 2003).
The major variations found in the Indian subcontinent and Chinese-origin rice germplasm are based on morphological, biochemical, and molecular aspects (Nadir *et al*.,2017). Furthermore, the estimation of genetic variation and relatedness among available germplasm is a vital aspect to be studied to conserve and maintain biodiversity and food security in the future (Ngangkham *et al*., 2019; Nybom *et al*., 2014). The

varietal identification and classification during breeding programs are measured using the distinctness, uniformity, and stability (DUS) approach, based on morphological descriptors (Tiwari *et al*., 2013). However, the determination of genetic variation based on morphological data/markers is inferior due to labour requirements, time consumption, and season-specific and agronomical traits highly influenced by environmental factors. In recent years, DNA fingerprinting has become a widely acknowledged approach for the identification of genetic differences and varietal purity between closely related cultivars to aid breeding programmes. It is a supplement to traditional morphological-based methods (Hebert *et al*., 2003) and is necessary for the protection of varieties under Plant Breeder's Rights Rules. Molecular markers are used in DNA fingerprinting to categorize breeding lines into several heterotic groupings and to distinguish between different kinds (Jamil *et al*.,2020a and b). In particular, it offers a reliable, economical, and environmentally friendly way to distinguish between plant genotypes and quickly reveal the genetic variety and variability among different species. The most potent methods for identifying plant genotypes and preventing the marketing of false and impure seeds are DNA markers (Nybom *et al*., 2014). Thus, the present investigation aims to assess the genetic variability and diversity at the molecular level among 11 rice varieties released by ICAR RC, Manipur Centre, and DNA fingerprinting using SSR markers.

Samples**:**The current study utilized 11 rice varieties that have been released from ICAR-RC NEH, Manipur Centre since 1993. All the 11 rice varieties are mentioned in the manuscript as RC Maniphou. The details of the 11 RC Maniphou rice varieties are summarized in **Table 1**.

Genomic DNA Isolation and PCR amplification: For genomic DNA isolation, the healthy seeds of the 11 rice varieties were sown in the pots. Young leaves of 21 days old seedling were collected and frozen in liquid nitrogen and then stored at -80°C deep freezer. The whole genomic DNA was extracted following CTAB method (cetyltrimethyl ammonium bromide) (Ngangkham *et al*., 2020; Doyle, 1990). The purified genomic DNA was checked for quantity and quality on 0.8% agarose gels. The DNA samples were diluted with nuclease-free water to the working concentration of 20 ng/µl for PCR amplification.

Table 1. List of rice varieties used in the experiment

S. No	Germplasm/ Variety	Year of released/ notification	Parentage	Agronomical traits
1	RC Maniphou-4 (RCM-7)	1993	Kalinga x Palman	Short duration variety (100-115 days) and suitable for pre-*kharif* (February sowing)
2	RC Maniphou-5 (RCM-8)	1993	Kalinga x Palman	Short duration variety (120-125) and suitable for pre-*kharif* (February sowing)
3	RC Maniphou-6 (RCM-5)	2005	CH 988 x IR-24	Medium short duration variety (125-130) and suitable for main *kharif*
4	RC Maniphou-7 (RCM-9)	2005	Mutant of Punshi (Gamma rays)	Short duration variety (135-140) and suitable for pre-*kharif* (February sowing)
5	RC Maniphou-10 (Lungnilaphou)	2005	Prasad x IR-24	Tolerant to neck blast and leaf blast, short duration variety (120-125) and suitable for main *kharif* (July-September)
6	RC Maniphou-11 (RCM-21)	2010	Prasad x IR-24	Resistant to Blast and tolerant to Brown plant hopper
7	RC Maniphou-12 (RCM-13)	2012	KD-2-6-3 (Leimaphou x Akhanphou)	Short duration variety (90-105) and suitable for early summer and pre-*kharif* (March-April sowing)
8	RC Maniphou-13 (RCM-30)	2016	KD-2-6-3 (Leimaphou x Akhanphou)	Medium duration fertilizer responsive high yielding variety (7-8 t/ha) and suitable for main *kharif* (June/July-October/November)
9	RC Maniphou-14 (RCM-33)	2022	IR 64 x Phougak	Medium duration, suitable for main *kharif* season, photo-insensitive with high yield potential (7-8 t/ha) and tolerant to leaf and neck blast, and false smut diseases
10	RC Maniphou-15 (RCM-36)	2022	KD-2-6-3 (Leimaphou x Phougak)	Medium duration fertilizer responsive high yielding variety (7.8 t/ha) and suitable for main *kharif* (June-July to October-November), resistant to leaf and neck blast diseases
11	RC Maniphou-16 (RCM-37)	2022	RCM-10 x RCM-9	Medium duration fertilizer responsive high yielding variety (7.3 t/ha) and suitable for main *kharif* (June-July to October-November), resistant to leaf and neck blast diseases

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The PCR mixture of 10 µl volume was prepared with the component containing: 5 µl of 2XDreamTaq Green PCR Master mix (Thermo Scientific, USA), 0.3 µl of each forward and reverse primers, 1 µl of diluted DNA (20 ng/µl), and 3.7µl of dd H_2O. The PCR program was set up by maintaining 94°C of 5 min for initial denaturation followed by 35 cycles of 94 °C for 45 sec, primers annealing for 45 sec at 55°C and elongation for 1 min at 72°C, which is followed by a final elongation at 72°C for 10 min. The PCR products were separated by gel electrophoresis in 3.5% Metaphor agarose gel (Lonza, USA) along with a 100-bp DNA ladder (DreamTaq, Thermo Scientific, USA) stained with ethidium bromide. The amplified bands were scored by documenting the image using Vilber E-box gel documentation system (Collégien, France).

Scoring of genotypic data and statistical analysis: A set of 42 SSR markers were used for genotyping of 11 RC Maniphou rice varieties. Detailed information on the markers is given in **Table 2**. When distinct bands in the anticipated size range were found during the PCR amplification of certain microsatellite markers in various genotypes, it was assumed that the amplification was successful; alternatively, the absence of the desired PCR band ranges was regarded as a null allele of the SSR marker.

The scored genotypic data of 42 SSR markers were used for the estimation of genetic diversity parameters *viz*., observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphism information content (PIC) and deviations from Hardy–Weinberg equilibrium (HWE) using the Cervus 3.0 program (Field Genetics Ltd, London, England). The Shannon's information index (I) and the effective number of alleles per locus (Ne), patterns of allelic richness, private allele frequency and F_{ST} (genetic differentiation) were calculated using GenAlEx 6.502 software (Peakall and Smouse 2012). The GenAlEx6.502 software was also used to compare the 11RC Maniphou rice genotypes and to estimate the pair-wise F_{ST} to partition genetic diversity and compute the PCoA (Principal coordinate analysis) by plotting the eigenvector values in a scatter graph taking the first principal component and the second principal component as the axes. The genetic relationship analysis was conducted by estimating genetic distance and similarity coefficients. An unweighted neighbor-joining un-rooted tree was constructed by the calculated NEI coefficient of dissimilarity index (Nei 1972) with a bootstrap value of 1000 using DARwin6 software (Perrier and Jacquemoud-Collet 2006).

Table 2. List of primers used in the study

Locus	Forward/Reverse Sequence	Chromosome No.
RM495	AATCCAAGGTGCAGAGATGG CAACGATGACGAACACAACC	1
RM6738	GTGCTTGCATGTGTAGTGGG CTCGTGCAAAAGGGTACTTG	1
RM 12160	ACGACGACCAATCCCAAGACG AGCAAATCGGAGTACAGGATCAGC	1
RM 12293	CAGGGCCTTAGACTTGGTCAGC CCAACAATCGAGGCGTATGTCC	1
RM 12404	TCTCACTCACTCACTACTTGCTTTGG ATGACAGCATCTCCTTGGATCG	2
RM 13775	CTGAGCTTCTGGTGCTTCCTAGC CATTATGAAGCATGAGGGCATCC	2
RM 14125	GGTACACAGTGGTAGGAAAGGTAGC GCAAGGTAGCAACCAAAGAATCC	2
RM 14320	CACCTGTAAATTAGGACACTGG CAGTGTACTTTGAACTGCCTAGC	3
RM 14379	ATGCAACAGGGAGGTGCTTGG TGAATCTGAAGATGCCCAAGAGG	3
RM 14981	GGCGAGCAGAAGTATAATCCAGAAGG CGCTTGTGGCTTACTGGCTTGG	3
RM 15539	CATGATGCATCGAGAATCTACCC AAGCGTTGTGTCTACCAACATGG	3
RM 16238	GCAGCGCATCATTGTATTAAGG GGACACTAAATCAGAAACCCATGC	3
RM 16341	TTCGGAGTCGGGTATAGGTAGTGC CTAAGGCTCGCAGGAGGAATATGG	4
RM 16730	ATCCCGAATCCAAATCCTTTCC AACGACGACAGCGAACAGAGC	4

Table 2. Continued..

Locus	Forward/Reverse Sequence	Chromosome No.
RM 17277	GGTCTCTCCTGCCTTGACACTCC AAGGTGGCATTGACTCACGAACC	4
RM 17599	GCGCCTGCTTCTTGATCTGC ATATCGACTCGGAGAAGGGAACG	4
RM 17803	AACTGCCATCTCTGAAACTCTGC CATCTCACTTCAGAAGGATCATAGCC	
RM 19039	GGACGCCATTGCGATCTCAGG CAGCAGCAGATCGTCCGTCAGG	5
RM 19303	GTGGCTTAACCACATGAGAAACTACC TCACATCATTAGGTGGCAATCG	
RM 7311	AGTGGTCGTTGAACTCGGAG TCGTGGCGCCTTTAATCTC	6
RM 20228	TTTCAGCATGACGCAGTTGTCC GATCCCATTGAGTATTGTACCTCACG	6
RM 20746	GCACCATCCGTCAGAAACAGC TCCGAGAAAGAAGAACCGCTAGG	
RM 20948	GCAAGCTGGAAGAACATCGTACC TGCTTATGGTTCTGGTCACTTCG	7
RM 21330	CTCATGCTTTCAGTCATTCAGTGC TCCTGGATTCATGGTGTCTTTAGC	7
RM 21776	TCGGGTATAATTATCGCAGCACACG ATGGATGGTACGAGGACGAGAGC	7
RM 22073	AAGAAGTTCTGCCTCAGCCAGTTCG CCTCCGTCGTCTCCTCCACTATCG	7
RM 22321	GAGATAGTGGTGGAGGTGGATGC GCACTTGTACTCCCATTTCTCAACC	8
RM 22881	CTAACGCTTTGCCTGCTTCTGC GTTGGGCCCATTACCACTCAGG	8
RM 23076	GTGTTCTGATGTTCCCTCTCTGC CCAACAAGGACTCACATGTCTCG	8
RM 23528	AGGCGGTGATGGATCTGGAGAGG CACCGGATCCAGGCAGATGG	8
RM 24071	TACTGAAGGCCAAGGAAGAGGTAGC GAGACTATGGTGTGGCGTCAATGG	9
RM 24309	CTTTCACCAGACTCCTCCTCACC CTGTAACCACATGCACCATCAGG	9
RM 25093	GATGGTAAAGGAAGAACGTGTGC CACTCATAGACGCATCACATAGCC	
RM 25262	CAATGCAAAGTCTTGTACGG GCTACATTGCATAGATCACTCG	10
RM 25675	TCTACCCAATCCACCCATCC AACAGGATGAGAAGAGAGATCAGC	10
RM 25866	TCTAACTCTGGCCATTAGTCCTTGG AAGTAGACGAGGACGACGACAGG	
RM 26033	AAGAGGACCTCGAGGATGTACCG CGTCAGCCTGTCTTGTTGTACTCG	11
RM 26603	GATTCCGATAGAACGGAAGAGAGC GAAGACCTCCTCACCAGTGAACC	11
RM 27096	AGTTAGGATCGCTTCCAGGTTCC TCCAACTGGAATATCGTCTTGTAGGC	11
RM 27363	ACTGCGTCCTCGTCACCTTCTGC CACTCCTCCGCCTTCTTGTACGG	11
RM 27487	CCAAGCACCACATTTGGTTTCC AACCTTGCTCAGCAGGACAGC	12
RM 27815	AACGCCCGCCTCTTTCTTCTTCC CCACCAACCAACGAACCAAACC	12

Multiplexing of SSR marker for DNA fingerprinting: Multiplex Polymerase Chain Reaction(Multiplex PCR) involves the simultaneous amplification of multiple target sites in a single tube using a different pair of primer sets in combination (Parida *et al*., 2020).In the present investigation, 11 primer pairs of polymorphic SSR markers which are found to be polymorphic among the 11 RC Maniphou rice varieties were used to make three different sets in combination based on the PCR product/ band size compatibility to develop a single PCR tube or a multiplex PCR system for differentiating all the 11 rice varieties developed by ICAR Manipur Centre, Imphal.

Being a staple food crop for the world population and considering the world population growth and diminishing arable land for cultivation due to soil degradation and global warming, the development of high-yield rice varieties with climate resilience is very important. The availability of genetic resources is the most important requisite for the development of any varieties and conservation of any germplasm. Determination of genetic diversity based on morphological and biochemical factors suffered various limitations as compared to the molecular markers due to the influence of the developmental stage and environmental conditions. With advanced genome sequencing facilities and the development of large number of DNA-based markers of rice genome, use of DNA fingerprinting based on molecular markers has been widely adopted for the identification of genetic differences, varietal purity between closely related cultivars to aid breeding programmes and to protect the varieties under Plant Breeder's Rights Rules. In this present study, an effort was made to determine the genetic diversity among the 11 rice varieties using the SSR markers and an attempt has been made to develop a DNA fingerprint dataset for 11 rice varieties for determination of genetic purity and variety identification.

Genetic Diversity Analysis: The present investigation aimed to detect the presence of genetic diversity among 11 varieties of rice (RM Maniphou series) released by ICAR-RC Manipur Centre since 1993. The study of genetic diversity provides useful information regarding relationship of available rice varieties/germplasm and to avoid the development of pest and disease in varieties due to narrow genetic diversity. Rice being the first crop plant to be sequenced for whole genome in 2005, large number of PCR based microsatellite markers or SSR markers were developed which have been extensively utilized in advancement of rice breeding programme throughout the world. The major advantages of SSR markers over the other PCR based markers is the genome-wide distribution, codominant inheritance, robust, reproducibility, suitability of high throughput genotyping. The number of alleles per polymorphic loci, number of effective alleles, observed and expected number of heterozygous alleles, Shannon's Index, and PIC value in the present investigation, are summarized in **Table 3**. A total number of 65 alleles were detected from 42 polymorphic SSR markers. The number of alleles per locus (K) ranged from 1 to 2 with an average 1.548. The markers RM 26033, RM 21330 and RM 20228 revealed the highest Observed heterozygosity (Ho) (1.00) followed by RM 16341 (0.919) and RM 16730 (0.714).

The Polymorphism Information Content (PIC) is generally used to determine the information content of a genetic marker. In the present study, the PIC value of markers ranged from 0 (RM263) to 0.375 with a mean of 0.163± 0.165 which demonstrates the moderate discriminatory power of these markers and their utility in analysis of genetic diversity, genetic conservation, etc. The highest PIC value was recorded by RM 16730 (0.375), RM 20228 (0.375), and RM 21330 (0.375) and these were considered the most informative markers among all the 42 primers. The maximum number of loci (80.09%) was found to be lower in observed heterozygosity (Ho) than expected heterozygosity (He) which might be due to selfing or self-pollination of rice. The mean value of expected heterozygosity and observed heterozygosity was 0.207±0.216 and 0.157±0.309 respectively. The markers RM16730, RM20228, RM21330 and RM26033 showed the highest number of alleles (2) with expected heterozygosity of 0.500 (H_E) indicating moderate heterozygosity which is similar to the other reports in rice germplasm (Choudhury *et al*., 2021; Ngangkham *et al*., 2019). Several genetic diversity and DNA fingerprinting studies were conducted on different crops using SSR molecular markers in popular Sorghum varieties of Tamil Nadu (Deshmukh *et al.,* 2013), in cotton (Santosh *et al.,* 2022) in groundnut (Amaravathi *et al.,* 2014) and using ISSR in Casuarina and *Allocasuarina* species (Chezhian *et al.,* 2009).

The effective number of alleles per locus (Ne) showed a range of 1.00 to 2.00 with an average of 1.372 ± 0.420. The Shannon's information index (*I*) ranged from 0 to 0.693 with a mean of 0.364±0.305 (**Table 3**). The overall low average gene diversity in the present study may be due to self-pollination, similar parentage of breeding lines and small number of rice samples studied.

Genetic structure and relationship analysis: All 42 SSR markers from 12 chromosomes of rice were used to infer the population structure and phylogenetic relationships among the 11 rice varieties. According to the Bayesian-based approach, by taking into account the peak value of ΔK, the optimal K values were determined using the STRUCTURE HARVESTER program. The optimal value of *K* and the number of clusters (*K*) was determined by plotting '*K*' against *ΔK* that exhibited a sharp peak at *K* = 4 which indicated the 11 rice varieties were distributed into four sub-groups (G1, G2, G3, G4). **Fig. 1.** shows the estimated value of k, which was found to peak at four. Similar results of two to eight subpopulations in rice were reported by Choudhury *et al*., 2021and Ngangkham *et al*., 2019. Population structure analysis in different

Table 3. Genetic diversity analysis for 42 SSR markers among 11 rice varieties released by ICAR-RC NEH, Manipur centre

Locus	Na	Ne	*I*	Ho	He	PIC	F_{ST}
RM495	2	1.198	0.305	0	0.165	0.152	1.000
RM6738	2	1.095	0.185	0.091	0.087	0.083	-0.048
RM 12160	1	1	0	0	0	0	#N/A
RM 12293	2	1.198	0.305	0.182	0.165	0.152	-0.100
RM 12404	1	1	0	0	0	0	#N/A
RM 13775	1	1	0	0	0	0	#N/A
RM 14125	1	1	0	0	0	0	#N/A
RM 14320	1	1	0	0	0	0	#N/A
RM 14379	2	1.862	0.655	0	0.463	0.356	1.000
RM 14981	2	1.658	0.586	0	0.397	0.318	1.000
RM 15539	1	1	0	0	0	0	#N/A
RM 16238	2	1.424	0.474	0	0.298	0.253	1.000
RM 16341	2	1.984	0.689	0.909	0.496	0.373	-0.833
RM 16730	2	2	0.693	0.714	0.500	0.375	-0.429
RM 17277	2	1.862	0.655	0.182	0.463	0.356	0.607
RM 17599	1	1	0	0	0	0	#N/A
RM 17803	1	1	0	0	0	0	#N/A
RM 19039	1	1	0	0	0	0	#N/A
RM 19303	2	1.984	0.689	0.182	0.496	0.373	0.633
RM 7311	2	1.923	0.673	0	0.480	0.365	1.000
RM 20228	2	2	0.693	1	0.500	0.375	-1.000
RM 20746	1	1	0	0	0	0	#N/A
RM 20948	1	1	0	0	0	0	#N/A
RM 21330	2	2	0.693	1	0.500	0.375	-1.000
RM 21776	1	1	0	0	0	0	#N/A
RM 22073	1	1	0	0	0	0	#N/A
RM 22321	2	1.984	0.689	0	0.496	0.373	1.000
RM 22881	1	1	0	0	0	0	#N/A
RM 23076	1	1	0	0	0	0	#N/A
RM 23528	2	1.198	0.305	0	0.165	0.152	1.000
RM 24071	2	1.528	0.530	0.222	0.346	0.286	0.357
RM 24309	1	1	0	0	0	0	#N/A
RM 25093	2	1.936	0.677	0.091	0.483	0.367	0.812
RM 25262	1	1	0	0	0	0	#N/A
RM 25675	1	1	0	0	0	0	#N/A
RM 25866	2	1.724	0.611	0	0.420	0.332	1.000
RM 26033	2	2	0.693	1	0.500	0.375	-1.000
RM 26603	2	1.342	0.423	0.100	0.255	0.222	0.608
RM 27096	2	1.936	0.677	0.455	0.483	0.367	0.060
RM 27363	2	1.600	0.562	0.500	0.375	0.305	-0.333
RM 27487	2	1.198	0.305	0	0.165	0.152	1.000
RM 27815	1	1	0	0	0	0	#N/A
Mean ± SD	**1.547±0.503**	**1.372±0.420**	**0.304±0.305**	**0.157±0.309**	**0.207±0.216**	**0.163±0.165**	

Na = Number of alleles, Ne = Number of effective alleles, Ho = Observed heterozygosity, He = Expected heterozygosity, PIC = Polymorphic Information Content. SD = Standard Deviation, I = Shannon's information index; F = genetic differentiation

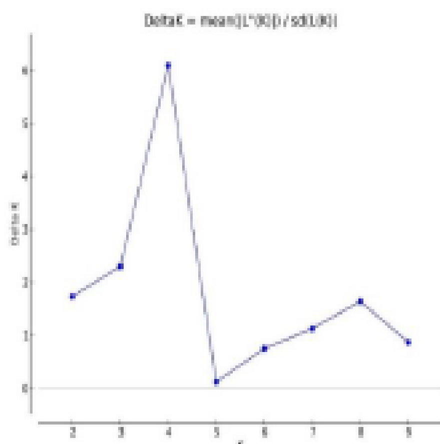

Fig. 1. Estimation of the maximum of adhoc measure ΔK determined by structure harvester was found to be k=4

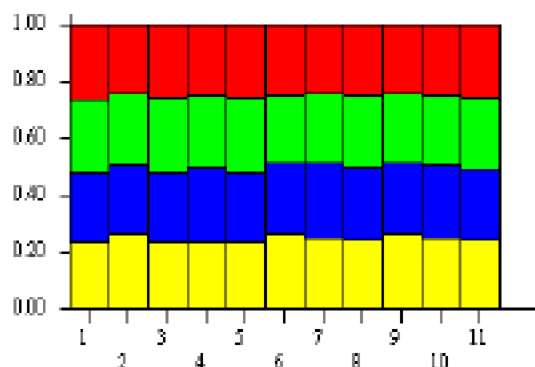

Fig. 2. Population structure of 11 rice varieties based on 42 markers. The maximum of adhoc measure ΔK determined by structure harvester was found to be *K* = 4, which indicated that the entire population can be grouped into four subgroups. Different colours within group indicates the proportion of shared ancestry with another group which has the same colour with the admixture. 1-11 represent 11 rice varieties listed in Table 1

rice diversity panels also revealed the existence of two to eight groups by Nachimuthu *et al*. (2015) and three subpopulations by Ngangkham *et al*. (2018). However, based on ancestry threshold of >70%, all the 11RC Maniphou varieties were classified into admixture.

All genotypic data based on the 42 SSR markers were used to infer the phylogenetic relationships among the 11 rice using DARwin5 software. On the basis of UPGMA method the 11 rice varieties were grouped into three major clusters (**Fig. 3**). Major cluster-I comprised only one variety, RC Maniphou 10, Major cluster-II included three varieties, which was further divided into two sub-groups II-A and II-B consisting of two varieties (RC Maniphou 11 and RC Maniphou 14) and one variety, RC Maniphou 5 respectively. Similarly, major cluster-III was further divided into two sub-clusters, III-A consisting of four varieties (RC Maniphou 16, RC Maniphou 7, RC Maniphou 13 and RC Maniphou 6) and III-B consisting of three varieties (RC Maniphou 15, RC Maniphou 12 and RC Maniphou 4). Among all the rice lines studied for cluster analysis, Maniphou 12 {KD-2-6-3 (Leimaphou) X Akhanphou} and Maniphou 15 {KD-2-6-3 (Leimaphou) X Phougak} showed the closest similarity which may be due to their similar lineage.

Multiplexing of PCR for Identification of RC Maniphou rice varieties: Harvesting of full yield potential of rice variety is largely depending upon the genetic purity of rice seed supplied to the farmers (Parida *et al*., 2020). Conventionally, GOT (grow-out test) is adopted to test the genetic purity of rice varieties which has many disadvantages such as time consuming, laborious, and the influence of environmental factors. With the advancement of genome sequencing technologies, many molecular markers have been developed for the rice genome which are fast, robust, reliable, highly accurate and free from environmental factors. Though with many advantages, the genotyping or DNA fingerprinting of plants with molecular markers comes with limitations of being costlier as compared to the conventional methods. The most feasible method is the use of multiplex PCR method which as superiority in terms of sensitive, fast identification in a single reaction and is economical over other conventional PCR based molecular markers (Satturu *et al*., 2018). Therefore, an attempt was made , for the first time , to develop a robust multiplex or single-tube PCR amplification system using 11 SSR primers to differentiate the 11 RC Maniphou rice series. The detailed forward/reverse sequence of the primers with their respective sets are presented in **Table 4**.

The amplified products were analysed by electrophoresis on 3.5 % Metaphor agarose gels and could distinguish all the 11 different samples from the three-tube multiplex PCR (**Fig.4**). All the 11 RC Maniphou could be differentiated among them through these multiplex PCR systems which could be useful to determine the genetic purity and variety identification.

DNA fingerprinting using SSR markers: Different molecular markers have been used to evaluate the fingerprints of different rice varieties and among them, SSR has been chosen as the best due to their co-dominant nature, reproducibility, abundance, polymorphic and distribution across the genome.

The lines exhibiting identical DNA profiles signify a close genetic relationship among them. In the current investigation, 42 SSR markers covering various chromosomes were used to get a fingerprinting profile

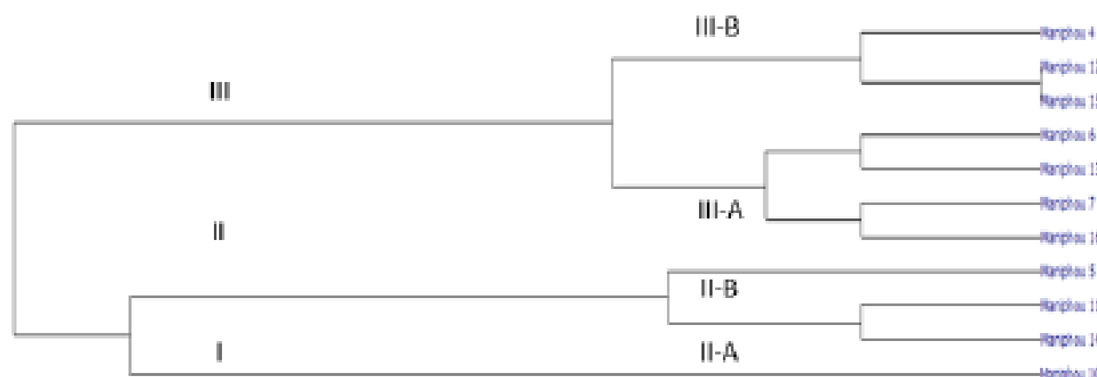

Fig. 3. Cluster analysis of 11 rice varieties based 42 SSR markers.

Table 4. List of polymorphic markers used in DNA fingerprinting

Set No.	Marker name	Primer sequence (5'-3')
Set I	RM 14379	Forward: ATGCAACAGGGAGGTGCTTGG Reverse: TGAATCTGAAGATGCCCAAGAGG
	RM 6738	Forward: GTGCTTGCATGTGTAGTGGG Reverse: CTCGTGCAAAAGGGTACTTG
	RM 7311	Forward: AGTGGTCGTTGAACTCGGAG Reverse: TCGTGGCGCCTTTAATCTC
	RM 27363	Forward: ACATATCGACGGTGGATGAGAGC Reverse: TCCGTGTGCATACATTCTTGAGC
Set II	RM 25093	Forward: GATGGTAAAGGAAGAACGTGTGC Reverse: CACTCATAGACGCATCACATAGCC
	RM 14981	Forward: GGCGAGCAGAAGTATAATCCAGAAGG Reverse: CGCTTGTGGCTTACTGGCTTGG
	RM 25866	Forward: GCCACATGGCAGCTTAATTATGAACG Reverse: CAATCGTCAAGCAACAAGCAAGC
	RM 27096	Forward: AGTTAGGATCGCTTCCAGGTTCC Reverse: TCCAACTGGAATATCGTCTTGTAGGC
Set III	RM 16730	Forward: ATCCCGAATCCAAATCCTTTCC Reverse: AACGACGACAGCGAACAGAGC
	RM7311	Forward: AGTGGTCGTTGAACTCGGAG Reverse: TCGTGGCGCCTTTAATCTC
	RM 17277	Forward: GGTCTCTCCTGCCTTGACACTCC Reverse: AAGGTGGCATTGACTCACGAACC
	RM25093	Forward: GATGGTAAAGGAAGAACGTGTGC Reverse: CACTCATAGACGCATCACATAGCC
	RM 22321	Forward: GAGATAGTGGTGGAGGTGGATGC Reverse: GCACTTGTACTCCCATTTCTCAACC

of the 11 rice varieties. Out of the 42 SSR markers, 11 SSR markers were found to be polymorphic while 31 were monomorphic. The SET I primers were able to distinguish between the identically sized amplicons of the RC Maniphou-6, RC Maniphou-7, RC Maniphou-12, RC Maniphou-15, and RC Maniphou-16 from the other varieties. The varieties RC Maniphou-4, RC Maniphou-13, and RC Maniphou-14 displayed various sizes, suggesting that the variations were distinct from one another. SET II primers were able to distinguish between the varieties RC Maniphou-12 and RC Maniphou-15 from RC Maniphou-6, RC Maniphou-7, and RC Maniphou-16 which the primers from SET I couldn't distinguish. Additionally, RC Maniphou-5, RC Maniphou-10, and RC Maniphou-11 exhibited variation based on the amplicon sizes of the SET II and III primers. SET III primers were also able to distinguish between RC Maniphou-7 and RC Maniphou-6 and RC Maniphou-16. **Fig. 5** displays a graphic representation of the fingerprinting profiles.

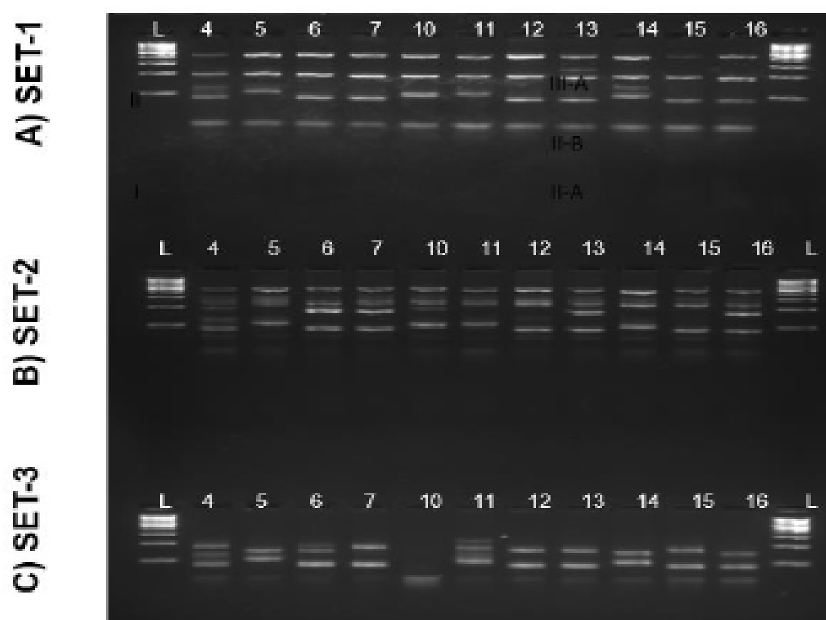

Fig. 4. Multiplex PCR for genotyping of 11 rice varieties, L- 100 bp ladder, 4- RC Maniphou 4, 5- RC Maniphou -5, 6- RC Maniphou -6, 7-RC Maniphou 7, 10- RC Maniphou -10, 11-RC Maniphou -11, 12-RC Maniphou 12, 13- RC Maniphou 13, 14- RC Maniphou -14, 15-RC Maniphou -15, 16-RC Maniphou -16

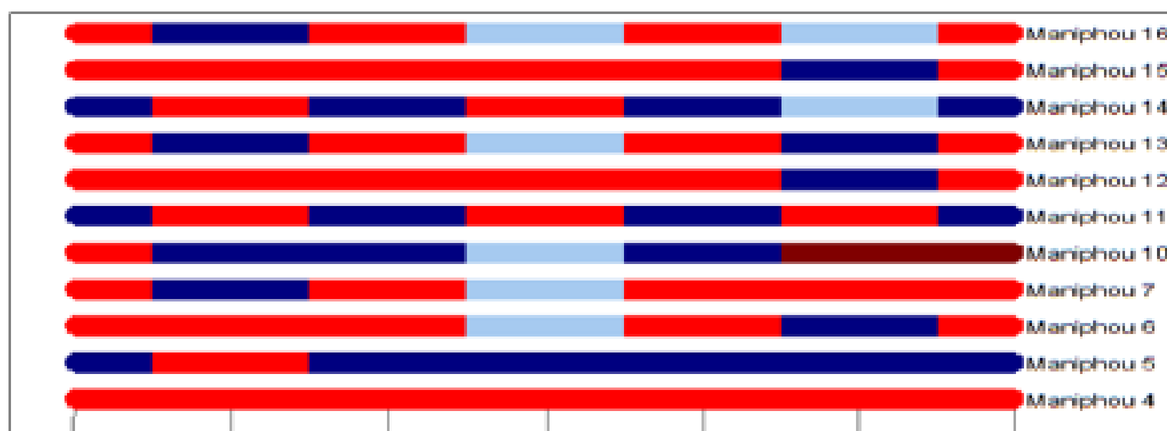

Fig. 5. Graphical representation of the fingerprinting profile of 11 RC Maniphou rice varieties

The present study on the genetic characterization of 11 popular rice varieties released by ICAR, NEH, Manipur Centre using SSR markers revealed the Maniphou 12 [KD-2-6-3 (Leimaphou) X Akhanphou] and Maniphou 15 [KD-2-6-3 (Leimaphou)X Phougak] showed the closest resemblance which might be due to similar parentages. A unique and clear separation of the different bands indicates the distinctness among the 11 lines while some lines had a similar genetic profile. The SSR markers displayed unique profiles that might be utilized to distinguish between the newly developed rice varieties from those that were previously cultivated by the farmers. The DNA fingerprinting profiles were capable of effective identification of rice varieties so far developed through multiplex PCR protocol. Furthermore, these selected primers could be useful for rice crop breeding.

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