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Molecular characterization of Jamir (*Citrus jambhiri*) accessions of Bangladesh through PCR based RAPD markers

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Abstract: Fourteen accessions of Jamir (*Citrus jambhiri*) were used to observe the variability among them by RAPD markers. Out of 16 screened decamer primers 6 primers were used which gave 80 clear and bright fragments. Of the 80 bands, 74 (92.5%) were polymorphic. Pair-wise comparison of DNA profile of the 14 Jamir showed inter-species similarity index (S_{ij}) value 86.45% for CJ-14 vs CJ-6 pair which was higher than all other accession pairs and the lowest was in between CJ-9 vs CJ-5 (30.58 %). The estimate of Nei's (1972) genetic diversity (I) for entire accessions of Jamir was 0.1488 and Shannon's information index (h) was 0.1951. The highest genetic distance (D) value 0.7444 was observed between the accession CJ04 and CJ10 and between CJ10 and CJ12. On the other hand, the lowest distance value 0.1193 was found among the accession CJ15 which revealed that there is a great genetic difference among the accessions. The UPGMA dendrogram produced 2 main clusters and more than 7 sub cluster of the 14 Jamir accessions. Thus, RAPD offer a potentially simple, rapid and reliable method to evaluate the variability among all species of plant.
Key words: *Citrus jambhiri*, PCR, RAPD markers

Introduction

Citrus jambhiri, belonging to the family Rutaceae, is locally known as Jamir. It is widely grown in most areas of tropical, sub tropical and border line of sub tropical or temperate climate (Lee *et al.*, 2004). It is very important in respect of its nutritional values especially being very rich in Vitamin-C. In Bangladesh mean intake of vitamin-C is far below from the recommended dietary allowance (Khan *et al.*, 2005) and 93% People are suffering from deficiency of Vitamin-C. Jamir can be eaten fresh, which can solve this Vitamin-C deficiency partially. It also contains some organic compounds which work against asthma, antidepressant, stress relief, aids digestion, colds, flue, fever, nosebleeds, mouth ulcers, throat infection and boils. Various kinds of food items like Jam, Jelly, pickle and salad can also be prepared from it.

World Citrus production and consumption have grown strongly since the mid -1980s (FAO, 2003a). Annual Citrus production of the world (from 1992 to 2002) grew at a rate of 2.3% and the growth is projected to be continued at a rate of 1.1% annually up to 2010 (Spreen 2001). Jamir grows every where in Bangladesh but the country stands a very low position in respect of production as well as yield of jamir. This is particularly due to lack of good quality and high yielding variety. In fact, until now there is no standard or recommended variety of jamir in Bangladesh. However, there are many landraces in our country, which are resistant against diseases, pests and adverse climatic conditions. After identification and study of specific character and their genetics, genes of interest may be transferred to any recognized variety. Moreover, characterization of our valuable landraces will help in protecting them from piracy by international companies or other countries and will establish our right on them. Therefore, it is inevitable to characterize and maintain the germplasm. Molecular markers have been able to analyze DNA directly, without any influence from the environment or tissue age (Tansksley *et al.*, 1989). Among the molecular markers, the RAPD (Random Amplified Polymorphic DNA) technique is widely used in Citrus because of their phenotypic neutrality and their ability to quick and easily reveals a large number of markers (Bastianel *et al.*, 1998). In Citrus, RAPD markers have been used for genetic mapping, identification of cultivars, hybrids, mutants, chimeras and phylogenetic analysis, since DNA fingerprinting using PCR-based markers are very important for breeding and taxonomy for Citrus. The present study was, therefore, undertaken to characterize the local Jamir accession through RAPD markers and to assess the genetic diversity and relatedness of Jamir accession for future breeding program.

Materials and Methods

The research work was conducted at the Field Laboratory, A total of 14 jamir accessions were investigated in this study. These accessions were maintained in the farm of "Collection, Molecular Characterization and Conservation of Landraces and Wild Relatives of Different Vegetables and Fruits of Bangladesh" project, at Horticulture Field Laboratory, Bangladesh Agricultural University, Mymensingh. DNA was extracted from 14 jamir accessions separately following standard protocol using SDS and Phenol: Chloroform: IAA followed by alcohol precipitation. DNA was confirmed using 1% agorose gel and quantified at 260nm spectrophotometrically. Out of 16 primers screened, 6 primers *viz.* OPA-05, OPA-07, OPA-10, OPC-05, OPK-07 and OPK-12 exhibiting good quality banding patterns and sufficient variability were selected by primer test for RAPD analysis. Polymerase Chain Reactions (PCR) were done in a volume of 10 µl containing 10 x PCR Buffer, 0.25 mM each of the dNTPs, 1µM of each of primer, 1 unit Taq DNA polymerase, 75ng template DNA and a suitable amount of sterile deionized water. Amplification was carried out in an oil free thermal cycler with the following thermal profile: initial denaturation step at 94°C for 3 min followed by 40 cycles at 94 °C for 1 min., 36°C for 41 min, and 72 °C for 2 min with 2 replications. PCR was confirmed by electrophoresis. All distinct bands (RAPD markers) were given identification numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0). The software DNA FRAG version 3.03 was used to estimate allelic length. This was used to estimate polymorphic loci, Nei's (1972) genetic distance (D), gene frequencies, gene diversity, Shanon information index (I) and to construct a Unweighted Pair Group Method of Arithmetic Means (UPGMA) dendrogram among populations using a computer program, POPGENE version

1.31 (Yeh *et al.,* 1999). The similarity index values (SI) between the RAPD profiles of any two individuals were calculated from RAPD markers according to the following formula: SI = 2 N_{xy} / N_x+ N_y, where N_{xy} is the total number of RAPD bands shared by individuals x and y, N_x and N_y are the number of bands in individual x and y, respectively (Lynch, 1990). Within population similarity (S_x) was calculated as the population of SI across all possible comparisons between individuals within a population.

Results and Discussion

Primer Selection: Among the 16 primers tested, 6 primers (OPA-05, OPA-07, OPA-10 OPC-05, OPK-07 & OPK-12) produced comparatively maximum number of high intensity bands with minimal smearing (Fig 1). Selected 6 primers generated 80 bands with size ranging from 79-2340 bp ranging from 10 to 18 and the average bands per primers was 13.33. Out of 80 bands, 74 bands (92.5%) were found to be polymorphic and 6 bands (8.11%) were found to be monomorphic. The primer OPA-07 produced the highest number of polymorphic bands (18). It showed a higher level of polymorphism. On the other hand the primers OPA- 05, OPA-10, OPC-05, OPK--07and OPL-12 generated 14, 10, 10, 13 & 13 polymorphic bands, respectively (Table 1).

Table 1. RAPD primers with corresponding bands score and their size range together with polymorphic bands observed in 14 Jamir accessions

Primer Codes	Sequences (5'-3 ')	Total number of bands score	Size ranges (bp)	Number of polymorphic bands	Proportion of poymorphic loci (%)
OPA-05	AGGGGTCTTG	14	259-2332	14	100
OPA-07	GAAACGGGTG	18	79-2190	18	100
OPA-10	GTGATCGCAG	10	245-2180	8	80
OPC-13	GATGACCGCC	10	264-2057	8	80
OPK-07	AGCGAGCAAG	15	232-1753	13	86.66
OPK-12	TGGCCCTCAC	13	350-2340	13	100
Total		80		74	546.66
Average		13.33		12.33	91.11

Frequency of polymorphic loci: Based on the presence and absence of bands, DNA polymorphisms were detected. Frequencies of maximum number of polymorphic loci were found to be high with the exception of OPA05-12, OPA07-1, OPA07-2, OPA07-11, OPA10-8, OPC05-6, OPK07-8, OPK07-14, OPK12-6 and OPK12-10 contain gene frequency 0.07 (Table 2). Though no accession-specific marker has been scored, the high level of polymorphism revealed by the proportion of polymorphic loci (93.88 %) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different accessions of Jamir.

Genetic variation: The Nei's (1972) genetic diversity for entire accessions of Jamir was 0.27 and Shannon's information index (I) was 0.40. There was a high level of genetic variation among the studied accessions of Jamir from the proportion of polymorphic loci point of view. Estimates of Nei's gene diversity (0.28) and Shannon information index (0.43) across all loci also support the existence of high level of genetic variation.

Inter-species similarity indices: The highest (79.33 %) and lowest (50.34%) level of inter-species similarity indices were generated by the primer OPA-10 and OPA-07, respectively (Table 3).

The Sij values within 14 Jamir accession ranged from 30.58 % to 86.45 % (data not shown). Pair-wise comparison of DNA profile of the 14 Jamir showed inter-species similarity index for CJ-14 vs CJ-6 pair (86.45%) was comparatively higher and for CJ-9 vs CJ-5 pair (30.58 %) was the lowest among all other accession pairs.

Genetic distance: The values of pair-wise comparisons of Nei's (1972) genetic distance (D) from combined data of the six primers ranged from 0.11193 to 0.7444 (data not shown). The highest genetic distance (0.7444) was observed between the accession CJ10 and CJ4 while the lowest genetic distance (0.1193) was observed between the accessions CJ15 (data not shown). The difference between the highest and lowest genetic distance indicated the presence of variability among the 14 Jamir accessions.

Table 2. Gene frequencies of polymorphic RAPD markers and summery of genetics diversity (h) statistics for all loci

RAPD markers	Number of Locus	Gene Frequency (Range)	Gene Diversity (Range)	
			(h)	(I)
OPA05	14	0.07 - 0.93	0.1327 - 0.4898	0.2573 - 0.6829
OPA07	18	0.07 - 0.93	0.1327 - 0.4898	0.2573 - 0.6829
OPA10	10	0.07 - 1.00	0.0000 - 0.5000	0.0000 - 0.6931
OPC05	10	0.07 - 1.00	0.0000 - 0.4898	0.0000 - 0.6829
OPK07	15	0.07 - 1.00	0.0000 - 0.5000	0.0000 - 0.6931
OPK12	13	0.07 - 0.93	0.1327 - 0.4592	0.2573 - 0.6518

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Table 3. Summary of band-sharing based on inter-species similarity indices (S_{ij}) generated by the primers

Primers	Inter-species similarity indices (S_{ij})
OPA-05	65.63%
OPA-07	50.34%
OPA-10	79.33 %
OPC-13	73.34%
OPK-07	70.01%
OPK-12	61.31%

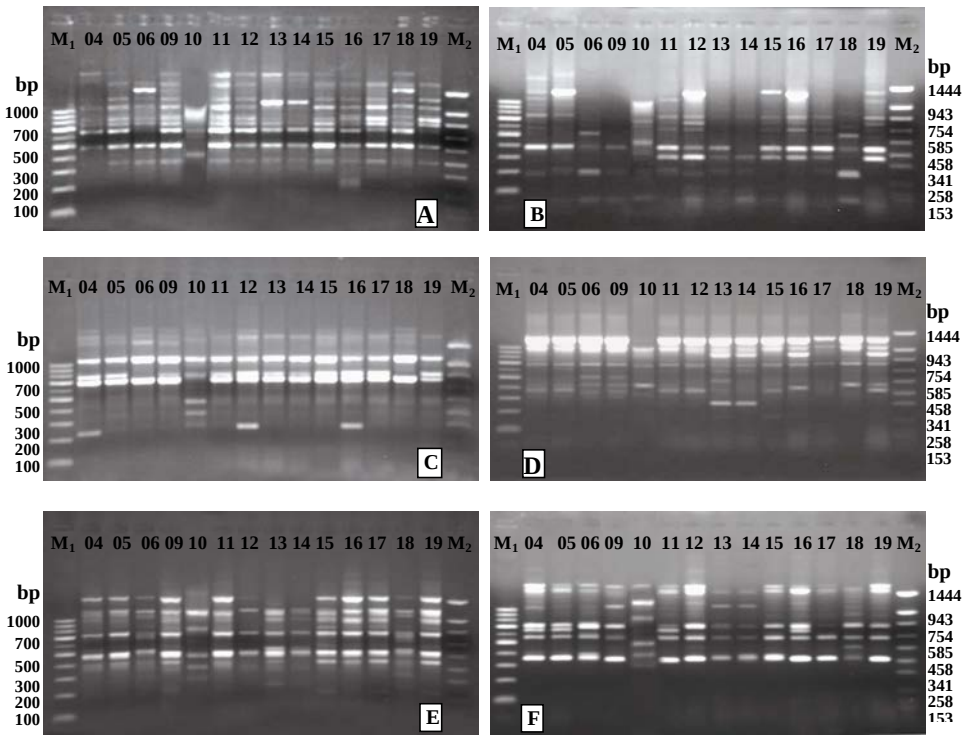

Fig 1. RAPD profiles of 14 jamir accessions using primer OPA-05 (A), OPA-07 (B), OPA-10 (C), OPC-05 (D), OPK-07 (E) and OPK-12 (F)**, [**M_1: 100 bp DNA ladder, M_2: pUC ladder]

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Phylogenetic dendrogram: UPGMA (Unweighted Pair Group Method of Arithmetic Mean) dendrogram (Fig 2) based on Nei's (1972) genetic distance indicated the phylogenetic relationship and segregation patterns among 14 Jamir germplasm of Bangladesh. The 14 Jamir germplasm grouped into two major clusters, **'a'** and **'b'**. Cluster **'a'** forming with only one Jamir accession (CJ10) collected from Sylhet. Other 13 Jamir accession were formed into cluster **'b'** which subsequently separated into two sub-clusters, **'c'** and'd'. Twelve Jamir accession formed the sub-cluster **'c'** which might be due to morphological and molecular distinctness from all other germplasm recources and to that of some extent of similarity within these 12 Jamir accessions. Sub-cluster 'd' formed with only one Jamir accession (Acc. No. CJ04) collected from Dinajpur. The sub-cluster **'c'** further separated into sub-cluters **'e'** and **'f'**. Sub-cluster **'e'** contained Acc. No. CJ13 and Acc. No. CJ14 which originated from Rajshahi whereas sub-cluster **'f'** again divided into sub-cluster **'g'** and sub-cluster **'h'**.

Here sub-cluster **'g'** contained Jamir germplasm Acc. No. CJ06, CJ09, and CJ18 all of them were collected from Mymensingh region. On the other hand sub-cluster **'h'** contained 7 Jamir accessions which were again divided

into sub-cluster **'i'**, **'j'**, **'k'**, **'l'** and so on. The germplasm resources of Jamir sourced from Mymensingh were found to be present in sub-cluster **'f'** supporting geographical similarity, although all the clustering patterns in the present study did not accord with geographical sources due to having genetically diversed germplasm resources. However, separation of the studied Jamir germplasms through major clusters could be explained by broad context of morphological, molecular as well as ecological divergences and formation of subsequent narrow distant sub-clusters could be explained by narrow range of diversification and comparatively strong relationship in respect of these factors within and between the Jamir germplasm resources.

Thus, it is found that, a wide variability was present among the collected accessions of Jamir. And the selection of superior genotypes from the collected accessions will be helpful to increase production through breeding program which in turn could improve varietal characteristics. The RAPD profiles of collected Jamir accessions of this study would be useful in monitoring genetic stability. However, further studies on molecular characterization using other molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) should be tried in order to generate markers for future breeding program.

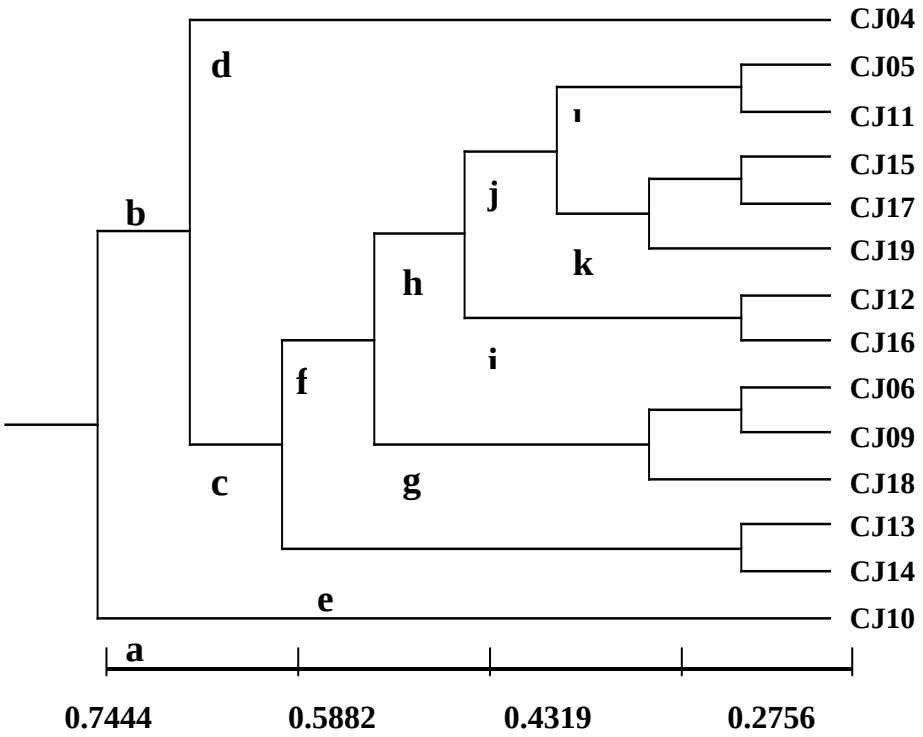

Fig 2: UPGMA Dendrogram based on Nie's (1972) genetic distance, summarizing the data on differentiation between 14 jamir germplasms according to RAPD analysis

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