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Assessment of Genetic Relationship among 15 Citrus Fruits Using RAPD

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ABSTRACT

Genetic relationship among 15 citrus fruits (one from the genus *Poncirus* and the rest from the genus *Citrus*) was assessed using Randomly Amplified Polymorphic DNA (RAPD) markers. Seven primers were selected after screening of 39 decamer primers which generated 91 clear and bright polymorphic bands. The Unweighted Pair-group Method using Arithmetic averages (UPGMA) dendrogram based on Nei's genetic distance segregated 15 citrus fruits into 2 main clusters where *Poncirus trifoliata* alone formed one cluster and the rest 14 species grouped together into another cluster. Inter-species similarity index and the UPGMA dendrogram based on Nei's genetic distance revealed that *Citrus megaloxycarpa* and *C. limonia* as the most closely related species among the 15 citrus fruits under study.

Key words: *Citrus* germplasm, interrelationship, molecular characterization, RAPD markers, similarity indices

INTRODUCTION

Citrus ranks first among the fruit crops in international trade in terms of value (UNCTAD, 2006; Uzun *et al*., 2009). It is one of the most economically important fruits and widely cultivated in subtropical and tropical regions of the world as well as in Bangladesh. As a part of centre of diversity, different Citrus fruits are cultivated in Bangladesh. Plant genetic diversity is a key component of any agricultural production system. An important component for effective and efficient management of plant genetic resources as well as their utilization is characterization of their germplasm. Such a characterization is essential not only for identification of species but also to determine genetic relatedness among them. The information generated could successfully be used in breeding programs wherever possible (Gulsen *et al*., 2010). This also assumes great relevance in the present context of intellectual property rights and trade agreements (Kumar *et al*., 2011).

Molecular markers have been able to analyze DNA directly, without any influence from the environment or tissue age (Tanksley *et al*., 1989). Among the molecular markers the RAPD (Random Amplified Polymorphic DNA) technique is widely used in Citrus because of its phenotypic neutrality and ability to quickly and easily revealing a large number of markers (Bastianel *et al*., 1998). RAPD is a PCR (Polymerase Chain Reaction)-based method that uses a short primer (usually 10 bases) to amplify anonymous stretches of DNA.

The genus *Citrus* represents mainly diploid species with a haploid chromosome number n-9 (Cameron and Frost, 1968) and easily crosses among themselves producing fertile hybrids (Barrett, 1985). Besides the natural crossing, spontaneous mutations, apomixes and selection are involved in determination of genes variability making the Citrus classification very complex (Herrero *et al*., 1996). Its complexity is also due to other factors such as incompatibility, high heterozygous sterility, depression by endogamy and polyploidy (Gmitter *et al*., 1996; Sahin-Cevik and Moore, 2011). Citrus breeding programs present biological limitations due to the heterogeneity of genus, polyembryonic nature, long reproduction cycle, sterility, incompatibility and endogenic depression.

Molecular marker has been evolved as a good way to study diversity and relatedness among Citrus germplasm. In Citrus, PCR-based markers have been used for genetic mapping (Cai *et al*., 1994) and to study genetic relationship among species or cultivars (Omura *et al*., 1993; Luro *et al*., 1995; Federici *et al*., 1998; Abkenar and Isshiki, 2003), to discriminate Citrus hybrids (Elisiario *et al*., 1999) and to identify Citrus mutants (Deng *et al*., 1995) and periclinal chimeras (Sugawara *et al*., 2002). It is inexpensive and fast, simple, represents the whole genome, requires a low concentration of genomic DNA and produces high polymorphic markers (Ferreira and Grattapaglia, 1998).

Reports on the study of Citrus germplasm in Bangladesh are not common. Farha (2005) attempted to characterize 14 Citrus fruits using morphological traits and isozyme markers. Identification and classification of Citrus cultivars based on morphological traits may often be hampered by low heritability and genotype×environment interaction (Nhan *et al*., 2003). Again isozyme analysis has its inherent disadvantages like limited number of enzyme loci and developmental and seasonal dependent enzyme expression. With the advent of techniques of molecular biology, DNA based markers have replaced enzyme markers in germplasm identification and characterization (Anand, 1998). Although, a number of researches have been conducted using advanced molecular markers to study citrus species in different parts of the world (Luro *et al*., 1995; Federici *et al*., 1998; Abkenar and Isshiki, 2003; Nhan *et al*., 2003; Biswas *et al*., 2009; Uzun *et al*., 2009; Amar *et al*., 2011; Gulsen *et al*., 2010; Sahin-Cevik and Moore, 2011), characterization of citrus germplasm of Bangladesh using any molecular marker is yet to be reported. In this study we used RAPD markers to characterize citrus fruit species with the aim to develop RAPD fingerprint for investigating the level of genetic relationship, characterizing and detecting polymorphism and diversity among 15 different Citrus fruit species of Bangladesh.

MATERIALS AND METHODS

Plant material: In order to carry out RAPD analysis, 15 citrus species viz., *Poncirus trifoliata, Citrus mitis, C. limonia, C. megaloxycarpa, C. assamensis, C. macroptera, C. jambhiri, C. medica, C. limettoides, C. variegata, C. limon,* C. aurantifolia, *C. sinensis, C. reticulata* and *C. grandis* were used in this study. Out of the 15 Citrus species, one (*P. trifoliata*) was from the genus *Poncirus* and rest 14 species were from the genus Citrus.

DNA isolation: For isolation of genomic DNA, actively growing young fresh leaf tissues were collected from each 15 species. DNA was isolated from leaf tissues as described by Murray and Thompson (1980). The confirmation of DNA was done in 1% agarose gel electrophoresis stained in ethidium bromide. The DNA concentration was determined using UV spectrophotometer

at 260 nm. A portion of the DNA was diluted to 25 ng μL^{-1} for use and both the stock solutions and diluted portions were stored at -20°C.

PCR amplification: Seven decamer primers, screened from 19 random primers of kits A, B, C, K (Operon Technologies, Inc), were used for PCR amplification. Amplifications of the samples were conducted in volumes of 10 μL reaction mix with 4 μL of genomic DNA as template, dilute primer = 2.5 μL, taq buffer = 1 μL, dNTPs (250 μM) = 1 μL, taq DNA polymerase = 0.2 μL and deionized water = 1.3 μL. DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf) programmed for 40 cycles of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, 2 min for extension at 72°C and a final extension at 72°C for 7 min. Amplified PCR products were separated on 1.4% agarose gel for 1.25 h at 120 V. Gel was stained in ethidium bromide solution and photographed using a gel documentation unit connected with a PC. A 100 base pairs DNA ladder (100 bp) and a pUC ladder were included in the gels as standard molecular weight markers.

Data analysis: Each RAPD product was assumed to represent a single locus and data were scored as the presence (1) or absence (0) of a DNA band. Only those fragment consistently amplified were considered for analysis. Genetic similarities were calculated according to the simple matching coefficient. Genetic similarity values defined as the fraction of shared bands between the RAPD profiles of any 2 individuals on the same gel according to the following formula:

$$\text{Similarity index: (SI)} = 2\ N_{xy}/N_x + N_y$$

where, N_{xy} is the number of RAPD bands shared by individuals x and y, respectively and N_x and N_y are the number of bands in individual x and y, respectively (Lynch, 1990; Chapco *et al*., 1992; Wilde *et al*., 1992). A simple, user friendly and time saver novel computation model was developed based on the aforementioned formula with Microsoft Excel to calculate the Inter Species Similarity Indices for this study. The genetic distances were determined from the dendrogram created based on the UPGMA (unweighted pair-grouped method using arithmetic averages) method (Sneath and Sokal, 1973) using a computer program POPGENE Version 1.31 (Yeh *et al*., 1999).

RESULTS AND DISCUSSION

Polymorphism percentage: Selected 7 primers generated 93 bands with size ranging from 186-1993 bp. Out of 93 bands almost all (99%) were polymorphic in nature (Table 1). This

Table 1: List of primers used with corresponding bands scored and their size range together with polymorphic bands observed in 15 Citrus fruits

Primer codes	Sequences (5′- 3′)	Total No. of bands scored	Size ranges (bp)	No. of polymorphic bands	Proportion of polymorphic loci (%)
OPA-05	AGGGGTCTTG	16	341-1993	15	93.75
OPA-O9	GGGTAACGCC	14	186-1500	14	100.00
OPA-10	GTGATCGCAG	14	250-1500	14	100.00
OPB-O1	GTTTCGCTCC	13	400-1594	13	100.00
OPC-O7	GTCCCGACGA	12	190-1500	12	100.00
OPC-13	AAGCCTCGTC	10	500-1844	10	100.00
OPK-12	TGGCCCTCAC	14	324-1500	14	100.00
Total		93		92	98.92

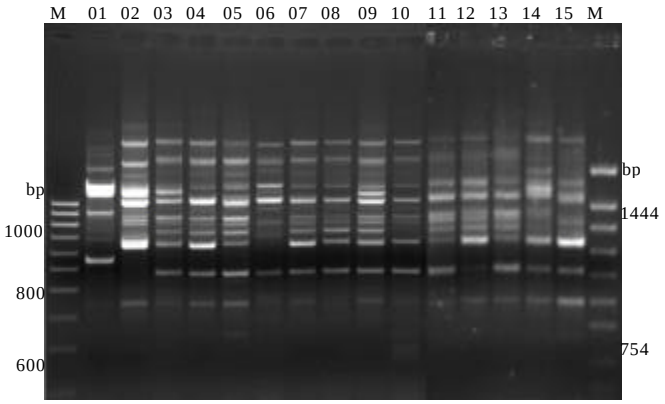

Fig. 1: RAPD profiles of Citrus species (01: *P. trifoliata*; 02: *C. mitis*; 03: *C. limonia*; 04: *C. megaloxycarpa*; 05: *C. assamensis*; 06: *C. macroptera*; 07: *C. jambhiri*; 08: *C. medica*; 09: *C. limettoides*; 10: *C. variegata*; 11: *C. limon*; 12: *C. aurantifolia*; 13: *C. sinensis*; 14: *C. reticulata*; 15: *C. grandis* using primer OPA-05. M: Molecular weight marker (100 bp DNA ladder in left side and pUC in right side)

proportion of polymorphism is much higher compared to some previous analysis of *Citrus* e.g., 65% polymorphism was found in 37 *Citrus* varieties by Nhan *et al.* (2003), 39% polymorphic bands was found by Andrade-Rodriguez *et al.* (2004) in 33 Volkamerian lemon. Heterozygous nature of Citrus species might be responsible for high level of polymorphism that resulted in the selection of large fragments from a chromosome or even an entire chromosome as explained by Luro *et al.* (1995). The seven different primers generated various banding patterns, ranging from 10 to 16. The primer OPA-05 produced the highest number of bands (16) and 15 of them (94%) were polymorphic. All other primers generated 100% polymorphic bands. The primer OPA-09, OPA-10 and OPK-12 generated 14 bands. Whereas, primer OPB-01, OPC-07 and OPC-13 produced 13, 12 and 10 bands, respectively. The banding patterns of 15 citrus species using primer OPA-05 are shown in Fig. 1 which exemplifies the typical RAPD banding patterns observed.

Relationship among the species: The inter-species Similarity Indices (SI) were estimated using the novel MS Excel based computation model much more quickly and easily compared to the manual calculation used in related previous studies (Rahman *et al.*, 2007; Biswas *et al.*, 2009; Mitra *et al.*, 2009) which can further be used for similar works with slight modification. The inter-species similarity index (S_{ij}) values of 15 citrus fruits ranged from 20.33 to 78.89% (Table 2, above diagonal). Pair-wise comparison of DNA profile of the 15 Citrus species showed inter-species similarity index for *C. megaloxycarpa* vs. *C. limonia* species pair was comparatively higher (78.89%) than all other species pairs. On the other hand, inter species similarity index for *C. grandis* vs. *P. trifoliata* (20.33%) and *C. limittoides* vs. *P. trifoliata* (21.06%) was the lowest among all the other species pairs. For the present study we developed an easy technique for calculation of similarity indices using an MS Excel model. Calculation of similarity indices using an MS Excel model is probably the first of its kind and not reported before in RAPD analysis.

Pair-wise comparisons of Nei (1972) genetic distance (D) between Citrus species were calculated from combined data for the seven primers and the values ranged from 0.18 to 0.82. The highest genetic distance (0.82) was observed in 2 species pairs i.e. *P. trifoliata* vs. *C. limittoides* and

Table 2: Summary of band sharing (%) based on similarity indices (S_{ij}) between individuals (above diagonal) and Nei's genetic distance (Nei, 1972) among 15 citrus species (below diagonal)

	P. trifoliata	*C. mitis*	*C. limonia*	*C. megaloxycarpa*	*C. assamensis*	*C. macroptera*	*C. jambhiri*
P. trifoliata	*	28.48	32.59	38.32	33.97	33.62	24.26
C. mitis	0.54	*	54.61	43.87	37.40	36.37	35.94
C. limonia	0.66	0.31	*	78.89	72.47	64.03	77.76
C. megaloxycarpa	0.64	0.42	0.18	*	75.79	59.09	68.44
C. assamensis	0.70	0.47	0.22	0.21	*	60.55	73.49
C. macroptera	0.66	0.54	0.34	0.42	0.37	*	71.09
C. jambhiri	0.82	0.49	0.18	0.31	0.21	0.26	*
C. medica	0.58	0.40	0.31	0.42	0.31	0.38	0.21
C. limettoides	0.82	0.35	0.29	0.40	0.29	0.45	0.25
C. variegata	0.66	0.40	0.34	0.42	0.34	0.47	0.26
C. limon	0.66	0.43	0.37	0.42	0.40	0.58	0.45
C. aurantifolia	0.70	0.50	0.40	0.45	0.37	0.62	0.45
C. sinensis	0.75	0.54	0.43	0.35	0.37	0.54	0.45
C. reticulata	0.66	0.34	0.34	0.45	0.40	0.50	0.42
C. grandis	0.77	0.42	0.32	0.37	0.32	0.42	0.31

	C. medica	*C. limittoides*	*C. variegata*	*C. limon*	*C. aurantifolia*	*C. sinensis*	*C. reticulata*	*C. grandis*
P. trifoliata	26.40	21.06	25.38	24.51	27.05	25.16	20.54	20.33
C. mitis	32.31	49.81	32.94	34.95	29.51	29.74	42.52	34.01
C. limonia	55.66	62.98	56.48	51.67	55.37	50.60	56.13	61.17
C. megaloxycarpa	48.12	53.29	52.95	48.67	52.42	55.61	43.74	55.98
C. assamensis	57.53	60.32	57.48	46.69	58.01	55.28	50.33	59.68
C. macroptera	52.45	55.09	43.17	36.59	41.22	43.03	43.94	53.58
C. jambhiri	67.86	67.39	65.20	44.50	51.24	49.49	49.29	62.35
C. medica	*	54.74	61.95	33.10	35.42	38.91	41.36	48.91
C. limettoides	0.32	*	51.02	56.36	27.31	35.38	38.81	29.18
C. variegata	0.22	0.35	*	35.78	34.54	47.41	43.97	49.73
C. limon	0.40	0.52	0.47	*	71.55	51.34	44.56	53.40
C. aurantifolia	0.47	0.45	0.54	0.19	*	61.54	48.97	66.20
C. sinensis	0.43	0.42	0.40	0.31	0.25	*	61.50	58.87
C. reticulata	0.37	0.32	0.37	0.37	0.37	0.34	*	68.32
C. grandis	0.32	0.34	0.35	0.35	0.26	0.32	0.21	*

P. trifoliata vs. *C. jambhiri*, followed by *P. trifoliata* vs. *C. grandis* (0.77) species pairs (Table 2, below diagonal). The lowest genetic distance 0.18 was observed in *C. megaloxycarpa* vs. *C. limonia* followed by *C. limon* vs. *C. aurantifolia* (0.19) species pair.

UPGMA dendrogram based on Nei (1972) genetic distance segregated 15 Citrus fruits into 2 main clusters (Fig. 2), where *P. trifoliata* alone produced cluster I and the rest 14 species grouped together and formed cluster II. It revealed that *P. trifoliata* was the most distantly related to all of the species compared. The result is similar to the cluster analysis conducted by Farha (2005) and Sawazaki *et al.* (1998). It was obvious that *P. trifoliata* alone formed a separate group as it came from a different genus, *Poncirus*. In cluster II, *C. mitis* alone formed sub cluster I and rest 13 species grouped together in sub cluster II. In sub cluster II; C. *limon, C. aurantifolia, C. sinensis, C. reticulata* and *C. grandis* produced sub-sub cluster II and rest 8 eight species produced sub-sub cluster I. In sub-sub cluster I, *C. macroptera* alone produced a single cluster and rest 7 grouped into another cluster. In UPGMA dendrogram, *C. limonia* was observed as close to the *C. megaloxycarpa* with the least genetic distance of 0.18. Result of the dendogram indicated

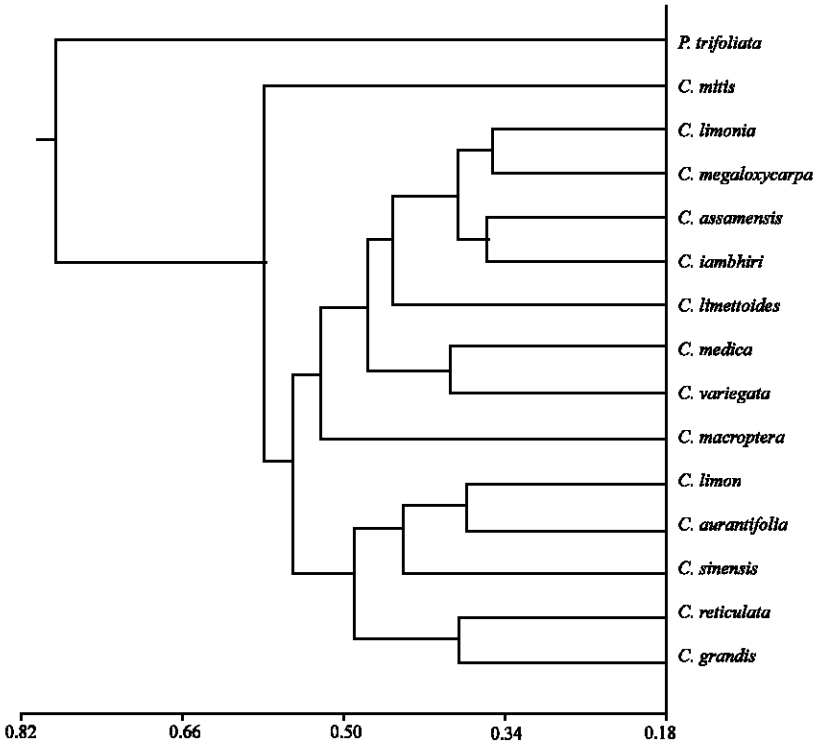

Fig. 2: UPGMA Dendrogram based on Nei's genetic distance of 15 *Citrus* species constructed by cluster analysis of RAPD markers

that *C. limonia* and *C. megaloxycarpa* probably related closely and remain in the same group of Citrus. *C. limon* and *C. aurantifolia* also clustered together with the genetic distance of 0.19. Highest genetic distance (0.82) was observed combinedly in *P. trifoliata* vs. *C. limittoides* and *P. trifoliata* vs. *C. jambhiri* species pairs and the lowest genetic distance (0.18) was observed in *C. megaloxycarpa* vs. *C. limonia* species of citrus fruits. The findings of the present study revealed the relatedness of 15 citrus species and emphasized the usefulness of molecular taxonomic analysis in genomic classification and genetic relatedness of citrus fruits which will be useful for better understanding of relatedness among the citrus and related species.

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