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ASSESSMENT OF GENETIC RELATIONSHIP AMONG FIVE MANDARIN CULTIVARS USING RAPD-PCR MARKER

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ABSTRACT: Genetic relationship among five mandarin cultivars was assessed using Randomly Amplified Polymorphic DNA (RAPD) markers. Five primers were selected after screening of many primers which generated 119 total bands. Clustering pattern analysis, the Unweighted Pair-group Method using Arithmetic averages (UPGMA) dendrogram based on Nei's genetic distance grouped five mandarin cultivars into two main clusters where S. mandarin and Ch. Mandarin formed one cluster and the rest three cultivars grouped together into two sub clusters, where C. mandarin and Sat. Mandarin formed one sub cluster and the other sub cluster included only B. mandarin cultivar. Inter-species similarity index (Dice and Jaccard) and the UPGMA dendrogram based on Nei's genetic distance revealed that *S. mandarin* and Sat. mandarin as the most closely related species among the five citrus cultivars under study.

Key words: *Mandarin* 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 center of diversity, different citrus cultivars are cultivated in Bangladesh. Mandarins (*Citrus* spp.) are the second most important group of citrus plants in the producer worldwide, with an annual production estimated as 760,000 tons, and a planted area of 31,000 ha, **(FAO, 2010)**. However, despite the high diversity presented by this group of citrus, only a small number of cultivars are used commercially.

The State of Rio Grande do Sul presents favorable climatic conditions for citriculture. These conditions, associated with a small properties' culture system (an average of two hectares) supply its market, but area, do not attend all needs satisfactorily. Moreover, the fact that the culture and its commercialization are restricted to few varieties, offered in a small period during the year, causes the need to import the product **(Bastianel, 1998)**. For these reasons, a Brazilian mandarin breeding program is essential to investigate the available genetic resources (species and varieties), and to supply the market demands. Several authors investigated and characterized morphologically different selections of citrus plants, into increase the number of genotypes with the potential to be used in breeding programs or to be released as new varieties. Characters related to plants, flowers, fruits and leaves were used by several authors **(Domingues *et al.*, 1999)** to describe and characterize distinct mandarin varieties and their hybrids. **Sartori *et al.* (1997)** determined the maturation period of the different mandarin genotypes used in the present work. The use of molecular markers has been a valuable and precise instrument to assist the genetic breeding of citrus species. Techniques like RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) were used in germplasm characterization, studies of genetic diversity, phylogenetic analyses, and systematic

(Coletta Filho *et al.*, 1998, 2000; Fang *et al.*, 1998; Federici *et al.*, 1998; Nicolosi *et al.*, 2000 and Hala El-Khayat, 2020). Microsatellites are sequences with one to four nucleotides, moderately repetitive in tandem, that are abundant in the euchromatin of vertebrates, insects and plants. They are considered ideal markers for genetic and physical genome mapping, identification and discrimination of genotypes, studies of population genetics, inbred line characterization, forensic studies, and medical genetics. In citrus, this marker has been used in studies of phylogenetic analysis and linkage **(Kijas *et al.*, 1995, 1997; Thomas *et al.*, 1998).** Therefore, in this study we used RAPD-PCR markers to characterize mandarin fruit species to develop RAPD fingerprints for the level investigating of genetic relationship, characterizing and detecting polymorphism and diversity among 5 different Mandarin fruit species in Egypt.

MATERIALS AND METHODS

Plant material: In order to perform RAPD analysis, five mandarin cultivars, Balady mandarin, Clementine mandarin (Tangerine), Satsuma mandarin, Santra mandarin and Chinese mandarin were used in this study.

DNA extraction: For extraction of genomic DNA, actively growing young fresh leaves were collected from each five cultivars. DNA was extracted from leaf tissues as described by **Murray and Thompson (1980).** The confirmation of DNA extraction was done using 1% agarose gel electrophoresis stained with ethidium bromide. The DNA concentrations were determined using UV spectrophotometer at 260 nm. A portion of the DNA was diluted to 25 ng/ µL for use and both the stock solutions and diluted portions were stored at -20°C.

PCR amplification: Five RAPD-PCR primers, 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 a 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% 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) 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 fragments 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:

$$\text{Similarity index (SI)} \quad 2 \quad N/N + NY$$

where, N, is the number of RAPD bands shared by individuals x andy, respectively and N, and NY are the number of bands in individual x andy, 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 Sakal, 1973)** using a computer program POPGENE Version 1.31 **(Yeh *et al.*, 1999).**

RESULTS AND DISCUSSION

Five Mandarin cultivars were subjected to further molecular fingerprinting analyses. RAPD-PCR based analysis revealed that the data for the five selected cultivars using five RAPD primers showed a total of 119 bands, 68% of them were polymorphic bands; the average number of amplified bands was 23.8 bands per primer (Table 1). All primers generated reliable polymorphic bands with all Mandarin cultivars (Figure 1). The RAPD primers achieved a rate of polymorphism ranged between 42.85% to 87% (Table 3) which successfully explains that RAPD primers are moderate in determining the genetic fingerprinting and discrimination of Mandarin cultivars due to their ability to produce polymorphic loci.

Table 1. List of primers used with corresponding bands scored and their size range together with polymorphic bands observed in 5 mandarin fruits

Primer code	Sequences (5'- 3')	Total bands	Polymorphic bands	Unique bands	Monomorphic bands	Rate of polymorphism (%)
OPA-02	TGCCGAGCTG	14	6	8	0	42.85 %
OPA-09	GGGTAACGCC	14	9	5	0	64.28 %
OPB-05	TGCGCCCTTC	31	27	4	0	87 %
OPD-01	ACCGCGAAGG	30	19	10	1	63.33 %
OPD-07	GGACCCAACC	30	20	9	1	66.6 %
Average		23.8	16.2	7.2	0.4	64.8 %
Total		119	81	36	2	

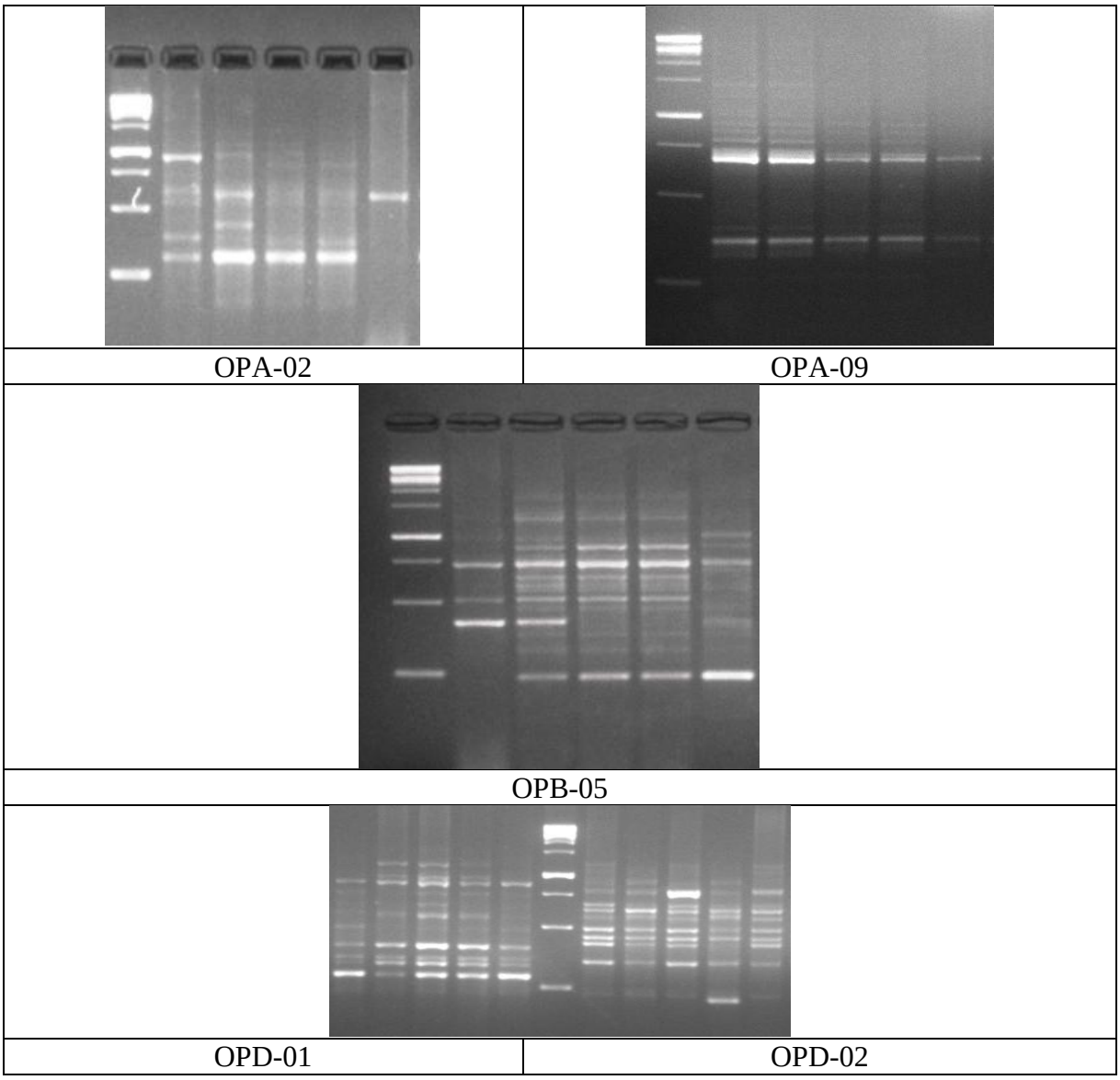

Figure 1: RAPD profiles of five mandarin species (B. mandarin, C. mandarin, Sat. mandarin, S. mandarin and Ch. mandarin) using five primers; OPA-02, OPA-9, OPB-05, OPD-01 and OPD-02, M=Molecular weight marker.

Table 2. Summary of band sharing (%) based on similarity indices (S.I between individuals) above diagonal) and Nei's genetic distance (Nei, 1972) among 5 mandarin species (below diagonal) by Dice

	B. mandarin	C. mandarin	Sat. mandarin	S. mandarin	Ch. mandarin
B. mandarin	1				
C. mandarin	0.38	1			
Sat. mandarin	0.24	0.38	1		
S. mandarin	0.26	0.52	0.56	1	
Ch. mandarin	0.17	0.29	0.54	0.36	1

Table 3. Summary of band sharing (%) based on similarity indices (S.I between individuals) above diagonal) and Nei's genetic distance (Nei, 1972) among 5 mandarin species (below diagonal) by Jaccard

	B. mandarin	C. mandarin	Sat. mandarin	S. mandarin	Ch. mandarin
B. mandarin	1				
C. mandarin	0.23	1			
Sat. mandarin	0.14	0.23	1		
S. mandarin	0.15	0.35	0.38	1	
Ch. mandarin	0.09	0.17	0.37	0.22	1

UPGMA dendrogram based on **Nei (1972)** genetic distance segregated 5 mandarin fruits into 2 main clusters (Fig. 2), where S. mandarin and Ch. Mandarin produced cluster I and the rest 3 species grouped together in two sub clusters which formed cluster II where B. mandarin was found alone in subcluster and both of *C. mandarin* and Sat. mandarin in the other subcluster. 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. megalox:ycarpa with the least genetic distance of 0.18. Result of the dendogram indicated that *C. limonia* and *C. megalox:ycarpa* 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 (0.56) was observed combinedly in *S. mandarin vs. Sat mandarin* pair and the lowest genetic distance (0.17) was observed in B. mandarin vs. Ch. mandarin species of mandarin fruits. The findings of the present study revealed the relatedness of 5 mandarin 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, **Coletta Fihlo *et al.* (1998)**.

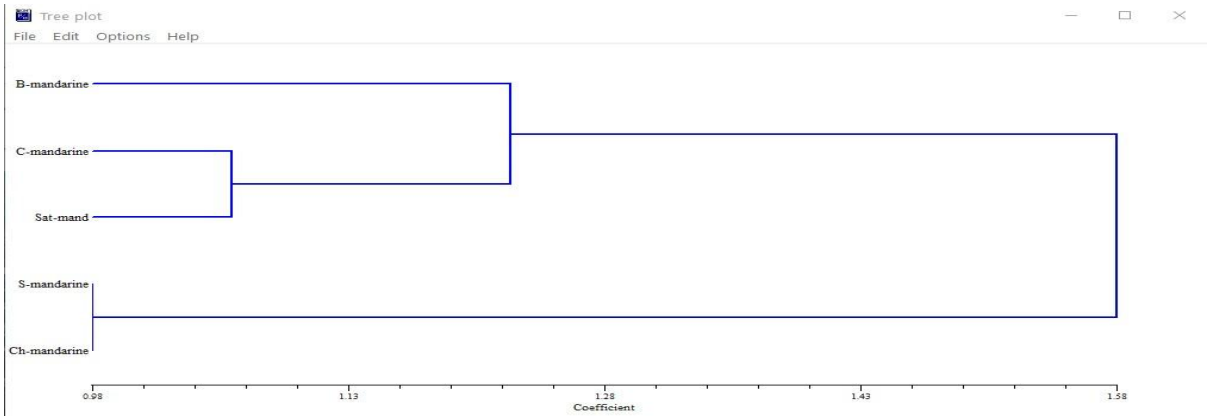

Fig. 2: UPGMA Dendrogram based on Nei's genetic distance of 5 Citrus species constructed by cluster analysis of RAPD markers

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