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Determination of Genetic Diversity among Local Mandarin Oranges (*Citrus reticulata*) Using Mature Seed Derived Calli through RAPD Markers

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Abstract

Orange (*Citrus reticulata*) is cultivated at several districts of Bangladesh but the genetic diversity among cultivars is still unknown. This research aimed to find out the diversity among locally grown nine mandarin orange (*C. reticulata*) cultivars. For this purpose, calli were initiated and used as a source of genomic DNA. Murashige and Skoog (MS) medium supplemented with phytohormones (2,4-dichlorophenoxyacetic acid, benzyl adenine, 1-naphthalene acetic acid, kinetin, indole-3-butyric acid) and malt extract were used for calli induction. DNA extracted from the calli of all the test cultivars' further subjected to PCR with ten RAPD markers, subsequently, data were assayed employing AlphaEaseFc 4.0 software, UPGMA (Unweighted Pair Group Method of Arithmetic Means), and the dendrogram was built by Statistica 7. The highest polymorphism information content (PIC) value was 0.79 for decamer OPX 04, and the lowest PIC value was 0.49 for both OPB 04 and OPB 17. From this study, the inter-relationship between nine cultivars was observed where Beanibazar, Bandarban, and Tamabil cultivars were closely related, followed by Jaflong and Karimganj cultivars. Companiganj and Darjeeling cultivars also shared some degree of genetic makeup. This study concludes that the used markers could be not only efficiently utilized in diversity analysis among *C. reticulata* cultivars but also for further improvement of local mandarin orange.

Keywords: Diversity; UPGMA; PIC; RAPD; Dendrogram.

1. Introduction

Mandarin orange is one of the most common *Citrus* fruits, broadly cultivated in the tropical and subtropical (35°N to 35°S) region. It has been classified to the genus *Citrus* and family Rutaceae and believes that it might have truly evolved in Australia, New Caledonia, and New Guinea [1]. Citrus fruit is grown exceeding 114 countries worldwide [2], and the diversification of *C. reticulata* took place in an area of Vietnam, Southern China, and Japan, and other secondary species came to light from the natural cross-pollination of four key taxa [3]. According to FAOSTAT [4], the global *Citrus* production in 2019 was 157.98 megatons which increased from 152.5 megatons in 2018. Bangladesh has a congenial climatic condition (mostly tropical) which is highly favorable for *Citrus* fruit production. In 2019, the total *Citrus* production in Bangladesh reported by FAOSTAT [4] was 165.327 kilotons. According to the reports, Bangladesh's northern and southern hilly districts are highly notable as *Citrus* growing regions. *Citrus* fruits are a vital source of human nutrition that boost the immune system to prevent diseases [5]. Mandarin orange is fortified with high content of vitamin C, carotenoids, dietary fibres, folate, flavonoids, etc. which can protect human health from cancers, oxidative stress, obesity and increase DNA stability [2, 6, 7]. Therefore, sluggish breeding of orange by orthodox methods, as well as cross-pollination, self-incompatibility, polyembryonic structure, and excessive heterozygosity makes it a challenging task for quality improvement [8]. Sometimes good quality producing cultivars were highly vulnerable to abiotic and biotic stresses [9].

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Molecular level is always a fundamental step for crop improvement that must take genetic variation into account nowadays [10]. Genetic diversity analysis is a main tool for assessing and developing genetic relatedness within germplasms, detecting various parental combinations for breeding programs as well [11-14]. Due to the rapid development of molecular genetics, different molecular techniques are being used to analyze DNA polymorphisms and draw a relationship among different cultivars to select the best and the fittest parents to stimulate the upgrading of existing crops because molecular data offer the authentic levels of diversity [15,16]. This enables the insertion of novel genes from elite genotypes into local genotypes, as well as sufficient parental plant selection to develop superior genotypes [17].

In *Citrus*, lots of molecular marker-based methods have been practiced to study genetic relationship; among them, RAPD (Random amplified polymorphic DNA) have been widely employed for molecular mapping [18], identification of varieties [19,20], hybrids [21], mutants, chimeras [22], and phylogeny inquiry [23]. Moreover, RAPD marker is able to detect polymorphism in a single PCR run [24]. Therefore, the current study is conducted to characterize nine local mandarin orange cultivars of Bangladesh using RAPD markers for better understanding of genetic variation.

2. Materials & Method

2.1 Plant materials

Calli were used to derive genomic DNA for the genetic diversity study, which were induced from mature seeds of the nine local mandarin orange cultivars retrieved from various orchards and markets in the Sylhet and Chattagram regions of Bangladesh and distinguished them based on their morphological analysis viz., average weight, average peel weight, average segment number, average seed number, and mean value of pH (Table 1).

2.2 Induction of calli

Freshly isolated, mature, healthy, dehusked seeds from mandarin orange fruits were used as explants on MS [25] basal media, which was selected based on numerous studies on *Citrus* and other plant species [26-30]. MS media fortified with various concentrations of 2,4-D and jointly with BA, KIN, NAA, IBA and malt extract (Table 2) [31,32]. Seeds were sterilized by a method described by Hasan et al. [33]. Sucrose (3%) was added as the carbohydrate source. Ten seeds from each cultivar were inoculated on each hormonal treatment. Visual observations were taken in every three days.

Table 1: List of collected local mandarin orange cultivars and their distinct characteristics used for evaluating diversity analysis.

Sl.	Cultivar	Sampling Location	Weight (avg.)	Peel weight (avg.)	Segment number (avg.)	Seed number (avg.)	pH (mean)
1	Beanibazar	Sylhet	85.40	15.43	10	19	3.40
2	Bandarban	Chattagram	109.64	18.69	10	14	3.86
3	Kinnow	Sylhet	146.96	33.20	10	14	3.81
4	Companiganj	Sylhet	131.35	27.60	10	18	3.69
5	Chhatak	Sylhet	121.99	34.29	11	11	4.16
6	Darjeeling	Sylhet	137.01	30.48	10	4	3.96
7	Jaflong	Sylhet	121.65	30.10	10	15	3.90
8	Karimganj	Sylhet	148.51	32.23	11	17	3.59
9	Tamabil	Sylhet	124.62	30.85	10	12	3.50

2.3 DNA extraction from calli

Only non-embryogenic calli induced by *in vitro* condition were used as a source of genomic DNA isolation by a modified CTAB method without liquid nitrogen [34]. About 0.2 g callus of individual cultivar was used for DNA extraction. Extracted DNA was stored at -20°C for further experimentation. Samples were confirmed for the presence of genomic DNA by agarose gel (0.7%) electrophoresis and pictured on a UV transilluminator [35]. Samples containing DNA that were confirmed via the above-mentioned method were used for subsequent analysis.

2.4 Selection of RAPD markers

Ten RAPD decamers were chosen on the basis of their polymorphic essence to measure genetic diversity among the collected cultivars [36-40]. These RAPD decamers were collected from Integrated DNA Technologies, Inc. (Coralville, IA, US) (Table 3).

2.5 PCR amplification

The amplification of the RAPD decamer through polymerase chain reaction was carried out with slight alterations of the thermal cycles stated by [41]. Total amplification volume was fixed at 25µl, with 12.5µl Master Mix (Promega Go Taq® G2 Green Master), 1.0µl RAPD primer, 2µl Dimethyl sulfoxide, 7.5µl nuclease-free water for each primer [35].

Table 2: Different hormone concentrations for calli induction on Murashige and Skoog medium.

Plant growth regulators	Concentration
2,4-D	0.5 mg/l
	1.0 mg/l
	1.5 mg/l
	2.0 mg/l
	2.5 mg/l
	3.0 mg/l
	3.5 mg/l
	4.0 mg/l
	4.5 mg/l
	5.0 mg/l
	2.0 mg/l + 0.75 mg/l
	3.0 mg/l + 0.75 mg/l
2,4-D + BA	2.0 mg/l + 0.50 mg/l
	3.0 mg/l + 0.50 mg/l
2,4-D + IBA	2.0 mg/l + 0.25 mg/l
	2.5 mg/l + 0.25 mg/l
	2.0 mg/l + 0.50 mg/l
2,4-D + KIN	2.0 mg/l + 0.75 mg/l
	2.0 mg/l + 0.50 mg/l
2,4-D + NAA	2.5 mg/l + 0.75 mg/l
	3.0 mg/l + 1.0 mg/l
BA	2.5 mg/l
	3.0 mg/l
	3.5 mg/l
2,4-D + Malt extract	2.0 mg/l + 0.05 g/l
	2.0 mg/l + 0.04 g/l
	4.5 mg/l + 0.04 g/l
	4.5 mg/l + 0.05 g/l
	1.5 mg/l + 0.04 g/l
	1.5 mg/l + 0.05 g/l

Table 3: Operon Random Primers (ORP).

Primer Code	Sequence (5′-3′)	GC content (%)	Tm value (˚C)
OPA01	CAGGCCCTTC	70	37
OPA02	TGCCGAGCTG	70	40.7
OPB04	GGACTGGAGT	60	32.2
OPB07	GGTGACGCAG	70	35.3
OPB17	AGGGAACGAG	60	33.1
OPC03	GGGGGTCTTT	60	33.1
OPF14	TGCTGCAGGT	60	35.7
OPL05	ACGCAGGCAC	70	34.8
OPX04	CCGCTACCGA	70	39.1
OPZ04	AGGCTGTGCT	60	37.4

Aeris™ Thermal Cycler was used to conduct PCR amplification. The thermal cycling conditions were set with an early denaturation at 94˚C for 5 minutes followed by 40 repeated series having three steps: denaturation at 94˚C for 60 seconds, annealing for 60 seconds, and extension at 72˚C for 2 minutes. The step for ending extension was synchronized at 72˚C for 10 minutes, and the hybridizing temperatures of primers with template were optimized on the basis of melting temperature (Tm) of individual primer. In addition, a holding step at 4˚C was set for making sure that the amplified products were kept stable [35]. Agarose gel (1%) electrophoresis (EDVOTEK EVT 300) was conducted, followed by ethidium bromide staining for analyzing the RAPD-PCR amplified products under an Ultraviolet transilluminator and snapped by a camera (Nikon D5300).

2.6 Alleles scoring

The gel images were used to analyze the bands and their patterns subsequently. By applying the program Alpha Ease Fc 4.0, molecular mass of individual amplicon was determined in base pair (bp). This program relates PCR fragments to specified fragment sizes of molecular weight markers (Generuler™ 1kb ladder). Identification numbers were then assigned to all the fragments or bands produced in gel location. Every amplified product of DNA was taken into account as a unit character by a given primer, and the amplified amplicons were recorded by a binary method as existent (1) or lacking (0).

2.7 Generated dataanalysis

The polymorphism information content (PIC) was assessed by the formula previously described by [42] utilizing a matrix produced by numeric figures.

$$PIC = 1-\sum Pab^2$$

Here, the ∑ (summation) signifies the total number of a precise decamer's occurrence for numerous alleles. Pab is the frequency of allele 'b' of decamer 'a', also, '∑' covers up to 'n' alleles [43]. In addition, pair-wise comparisons among the samples were conducted based on the existence or nonexistence of distinctive and mutual PCR fragments and further applied to produce a similarity matrix. Estimates of genomic similarity (F) were taken into consideration to calculate between all pairs of the samples [35, 42].

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

Where N_x = sum of amplified PCR products reported by cultivar 'x', N_y = sum of amplified PCR products reported by cultivar 'y' and N_{xy} = the frequency of mutual PCR products of cultivars 'x' and y'. The subsequent similarity coefficients were utilized to estimate the interactions among the orange cultivars with a group inquiry by an unweighted pair-group method with arithmetic mean (UPGMA). The results were used to make a dendrogram applying Statistica 7 software [35].

3. Results and Discussion

3.1 Callus induction

Both yellowish and whitish calli started to grow after 25 days of inoculation and embryogenic calli (EC), as well as non-embryogenic calli (NEC), were observed. When the calli grown approximately the size of 2 cm (in width) (Figure 1), they were removed from the media and used for DNA extraction. [58] have reported that the EC calli have genetic integrity and produce true to type plants through somatic embryogenesis without the exertion of selective pressure and confirmed by ISSR markers.

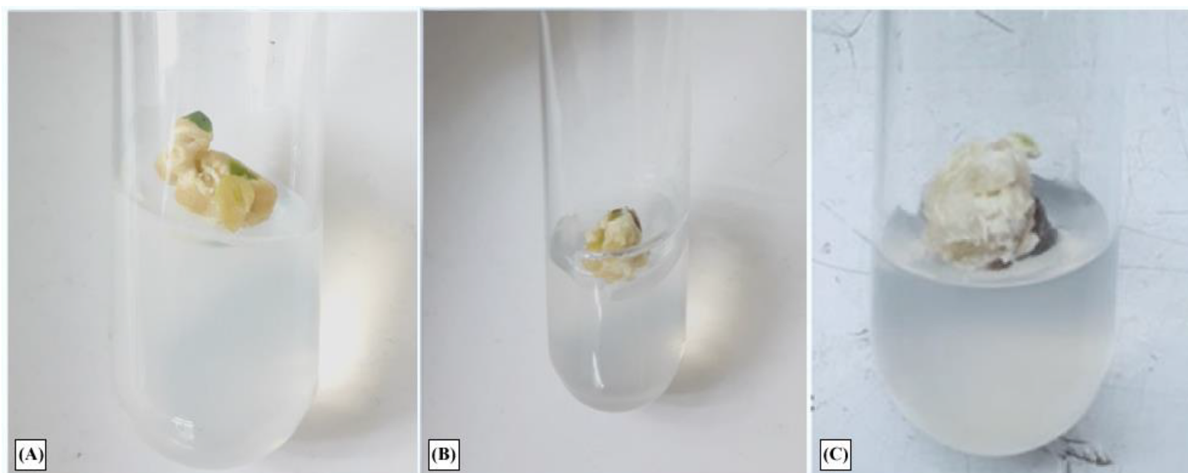

Figure 1: Callus of (A) Beanibazar, (B) Bandarban and (C) Karimganj cultivar on MS media

3.2 Polymorphism analysis of RAPD markers

RAPD-PCR technique was followed in this study since its simplicity, rapidity, sensitivity, no prior knowledge on genome was mandatory and this technique could produce many DNA segments as amplified products in a single run. The polymorphic RAPD markers reported by researchers were utilized for amplification of particular cultivars through PCR which generated heterogeneous PCR amplicons. The total amplicon number was 236 (Table 4). These RAPD decamers produced bands with diverse lengths from 300bp to 2920bp. Among the 236 bands, 119 bands were polymorphic, and 117 bands were monomorphic.

Among all the markers, OPX 04 produced the maximum number of polymorphic bands (33 bands; Figure 2), followed by OPZ 04 (22 bands), OPB 07 (19 bands) and OPA 01 produced no polymorphic bands at all (Table 4), The highest number of monomorphic bands, 18 was reported by five markers, namely OPA 01, OPA 02, OPC 05, OPF 14 and OPL 03. On the other hand, marker OPB 07 did not showed monomorphic band (Table 4).

The value of Polymorphism Information Content (PIC) represents the allelic variation among cultivars [44-49]. The current study found the range of PIC values from 0.49 (OPB 04) to 0.79 (OPX 04) with an average value of 0.635 (Table 5). This low level of polymorphism indicated that the mandarin cultivars were closely related. The average PIC value was somewhat similar to the report of [24], but the lowest and maximum PIC values differ. Nevertheless, the average PIC value of 0.635 signifies the fact that the molecular markers used to detect variation in this study were quite efficient in the detection of polymorphism between the different species of mandarin orange.

Two markers, OPB 07 and OPX 04 showed the highest (100%) polymorphism, whereas OPA 01 and OPF 14 showed no polymorphism among the genotypes. The highest number of bands per cultivar was 3.67, which were amplified by the primer OPX 04, and the lowest number of bands was 1.78 per cultivar, which was amplified by the primer OPB 04 and OPB 17. Among the RAPD primers, OPA 01 is a common primer used in previous studies for genetic variability of different Citrus species [19, 24, 50, 51]. Interestingly, the total number of bands produced by OPA 01 in this study (18) was identical to the reports of [24]. Although, bands produced by OPA 01 in this study were monomorphic but [24] reported 100% polymorphic bands for the same marker. The use of the other nine selected RAPD markers for the genetic variability study of mandarin orange was somewhat unique. Ten RAPD primers showed a significant level of polymorphism, 49.57% between these nine cultivars, which was comparatively lower than the previous reports [24, 50].

Table 4: Number of total bands along with polymorphic and monomorphic bands produced by the RAPD primers, band sizes and PIC value observed in local mandarin orange cultivar

Primer	Size of DNA bands (bp)	Total no. of DNA bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (%)	No. of bands per variety	PIC value
OPA 01	390-1060	18	0	18	0	2	0.50
OPA 02	710-1580	25	7	18	28	2.78	0.65
OPB 04	520-1040	16	7	9	44	1.78	0.49
OPB 07	460-1900	19	19	0	100	2.11	0.72
OPB 17	470-1290	16	7	9	44	1.78	0.49
OPC 05	430-1590	31	13	18	42	3.44	0.76
OPF 14	330-490	18	0	18	0	2	0.50
OPL 03	370-950	29	11	18	38	3.22	0.71
OPX 04	310-2920	33	33	0	100	3.67	0.79
OPZ 04	300-1110	31	22	9	70	3.44	0.74
Total		236	119	117			
Average		23.6	11.9	11.7			0.635

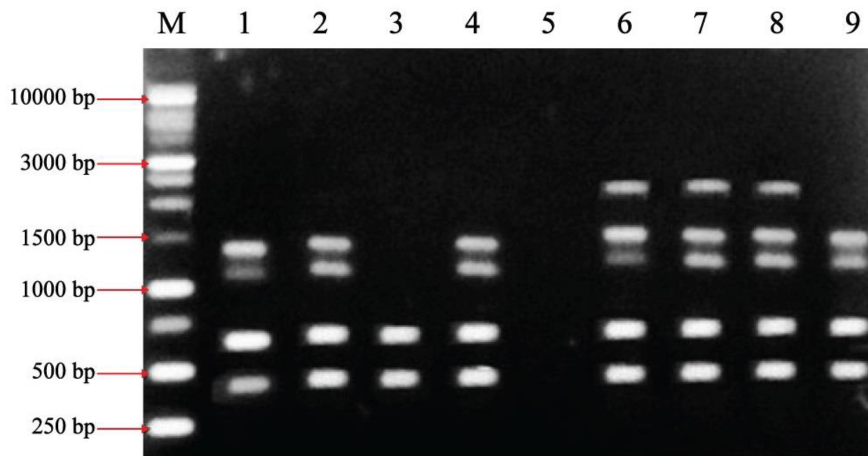

Figure 2: DNA profile of nine mandarin orange cultivars with RAPD primer OPX 04. Here, Lane M: Molecular weight marker (Generuler™ 1 kb ladder); Lane1: Beanibazar cultivar, Lane 2: Bandarban cultivar, Lane 3: Kinnow cultivar, Lane 4: Companiganj cultivar, Lane 5: Chhatak cultivar, Lane 6: Darjeeling cultivar, Lane 7: Jaflong cultivar, Lane 8: Karimganj cultivar and Lane 9: Tamabil cultivar.

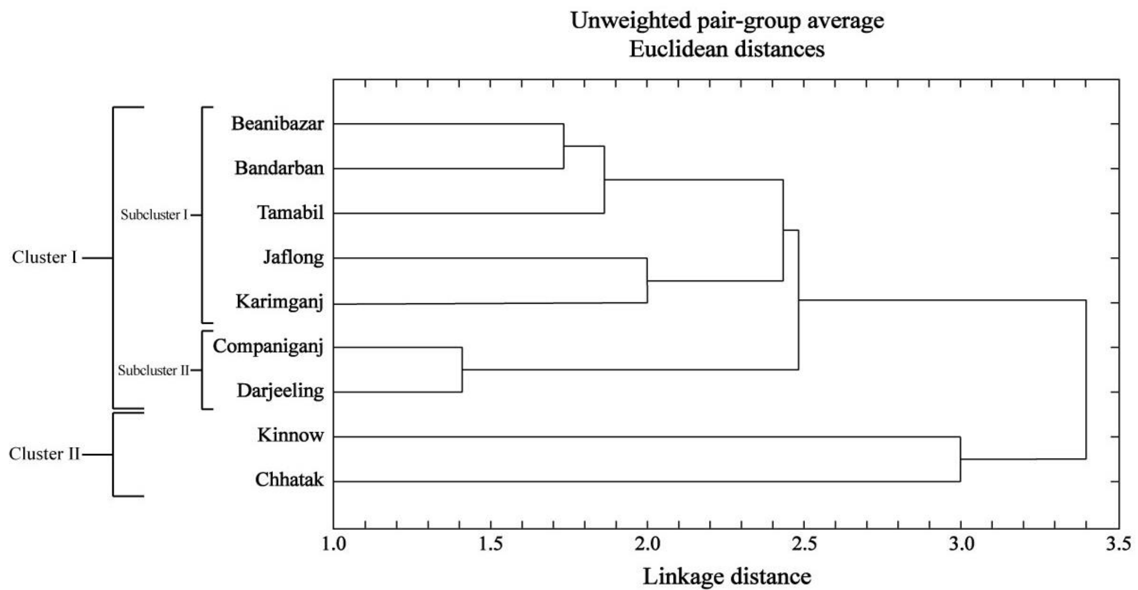

Figure 3: UPGMA dendrogram of nine local mandarin orange genotypes.

Table 5: The pair-wise inter variety similarity indices (S_{ij}) among nine local mandarin orange genotypes

Germplasm	Beanibazar	Bandarban	Kinnow	Companiganj	Chhatak	Darjeeling	Jaflong	Karimganj	Tamabil
Beanibazar	1								
Bandarban	0.95	1							
Kinnow	0.79	0.78	1						
Companiganj	0.91	0.89	0.8	1					
Chhatak	0.77	0.79	0.77	0.73	1				
Darjeeling	0.87	0.86	0.77	0.96	0.7	1			
Jaflong	0.9	0.89	0.73	0.88	0.75	0.88	1		
Karimganj	0.86	0.88	0.71	0.91	0.72	0.91	0.93	1	
Tamabil	0.93	0.95	0.75	0.91	0.81	0.91	0.93	0.93	1

3.3 Likeness indices within a cultivar and between cultivars

Genetic distance was determined by calculating resemblance or variances between two genotypes compare to the incidences of a specific trait. The degree of the kinship between orange genotypes was estimated by forming a similarity matrix (Table 5). It was formed calculating the number of common RAPD amplicons.

The pair-wise assessment of likeness in cultivar was 1. The pair-wise estimate of likeness between cultivars ranged from 0.70 to 0.96. The highest inter variety similarity indices was found between Companiganj and Darjeeling cultivar, which was 96%, and the lowest inter variety similarity was observed between Chhatak and Darjeeling cultivar, which 70%.

3.4 Cluster analysis

A cluster diagram was created by using genetic similarities found in RAPD data. UPGMA was applied to build up a cluster to measure the phylogenetic relationships among various local mandarin orange cultivars. The UPGMA clustering system divided nine mandarin orange cultivars (Figure 3) into two clusters, where Cluster I was further distributed into two subclusters. Subcluster I contains five mandarin orange cultivars, Beanibazar, Bandarban, Tamabil, Jaflong and Karimganj; Subcluster II mandarin contains two mandarin orange cultivars, Companiganj and Darjeeling. Cluster II has only two cultivars, namely, Kinnow and Chhatak. The linkage distance between the two clusters (3.4) was significant.

From this analysis, it was observed that Kinnow and Chhatak cultivars were distantly related to the rest of the seven cultivars, whereas the cultivars of Companiganj and Darjeeling were closely akin. The lowest linkage distance in a cluster was 1.4, which was between the cultivars Companiganj and Darjeeling in Subcluster II in Cluster I. The maximum linkage distance in a cluster was observed to be 3.0 between the cultivars Kinnow and Chhatak in Cluster II. The linkage distance between the cultivars Beanibazar and Bandarban was a little more than 1.7, and between the cultivars, Jaflong and Tamabil is 2.0. The linkage distance between the Tamabil cultivar and the cluster of Beanibazar and Bandarban cultivar was close to 1.9; the linkage distance between the cluster formed between these three cultivars and the cluster of Jaflong and Karimganj cultivar is a little above 2.4. The linkage distance between Subcluter I and Subcluter II is 2.5.

Among the nine cultivars, only the Kinnow (*Citrus nobilis* Lour × *Citrus deliciosa* Tenora) cultivars ancestral origin was known [52, 53]. The two clusters point to the divergence in the origin of the *Citrus* cultivars. Kinnow was probably the only cultivar in this study that widely cultivated in South Asia Himalayan foothills reasons including northern Afghanistan, Pakistan, India, Nepal and Bhutan, etc [53]. Most of the mandarin oranges belong to the *Citrus reticulata* species as stated by an array of different studies on mandarin oranges, but some mandarin oranges like Kinokuni Mandarin Orange and Satsuma Mandarin Orange belong to other species of the genus *Citrus* [54-57].

The divergence of the dendrogram into two distinctly separate clusters was likely due to the fact that the Kinnow mandarin orange is not a *Citrus reticulata* species, rather a crossbreed of two different *Citrus* species [52]. The cultivar Chhatak is closely related to Kinnow as compared to the other cultivars, which the UPGMA dendrogram can conclude. This suggests that the cultivar Chhatak is likely not a member of the *C. reticulata* species. The clustering of the other seven cultivars in a cluster with a small linkage distance suggests high genetic similarity and homogeneity between these cultivars, but there is insufficient information available to determine their pedigree.

5. Conclusion

This study confirms the efficiency of RAPD markers as an excellent tool for studying the genomic diversity of *Citrus reticulata*. The mandarin orange accessions in Bangladesh have a diversified genomic background. The nine cultivars have a significant amount of polymorphism between them. The data generated by this study will help in facilitating breeding programs to develop mandarin orange varieties with novel traits, more suitable for fending off attacks from different pests and tolerant to abiotic stresses. The data generated by this study will also help breeders and researchers alike in delineating the ancestral lines of the local cultivars farmed in Bangladesh, which is of utmost importance as *Citrus* species around the globe that have agricultural importance are hybrids or crossbreeds. This will help in guiding further development of mandarin orange cultivars. Most of the collected accessions were landraces; thus, the study will also aid in the conservation of the germplasms of these accessions. Though further study is an absolute requirement for generating QTL and linkage maps of these accessions, this study will surely aid in steps and procedures, resulting in future sustainability in farming local mandarin orange in Bangladesh.

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