Genetic Variability and Relationship Studies of Mandarin (*Citrus reticulata* Blanco) Using Morphological and Molecular Markers

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Abstract Mandarin (*Citrus reticulata* Blanco) is the second most important citrus crop worldwide. A total of 25 mandarin accessions collected from North, Central and North-Eastern regions of India, were evaluated for phenotypic and genotypic variations using morphological and random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. Significant variability was observed in the selected mandarin accessions by the analysis of eleven quantitative and 22 qualitative morphological characters of leaves, fruits and seeds. RAPD and ISSR markers also showed polymorphism (95 %). Jaccard's genetic similarity value of RAPD and ISSR was found in the range of 0.46–0.96 (average 0.71) and 0.42–0.96 (average 0.69), respectively suggesting moderate level of genetic diversity within the mandarin group. Four of the fifteen RAPD primers were also able to generate cultivar specific amplicons, which may be used for identification of cvs. SZ-In-Com, Dancy, Fairchild and Fewtrell's Early. The present study revealed that morphological and molecular markers can be successfully utilized for determining genetic diversity and genetic relationship of mandarin group and used in mandarin breeding programmes.

Keywords *Citrus* germplasm · Genetic diversity · Polymorphism · Cluster analysis · Molecular markers

Introduction

Citrus L. belongs to the sub-family Auarantioideae of the family Rutaceae and one of the most important commercially cultivated fruit crops in the world [24]. It includes some of the major fruit crops, such as *C. aurantifolia* (Christm.) Swingle (lime), *C. limon* (L.) Burm. f. (lemon), *C. maxima* (Burm.) Merr. (pummelo), *C. medica* L. (citron), *C. paradisi* Macf. (grapefruit), *C. reticulata* Blanco (mandarin) and *C. sinensis* (L.) Osbeck (sweet orange). Mandarin group is comprised of numerous species as well as inter-generic and interspecific hybrids which made them the most

phenotypically heterogeneous of the genus *Citrus*. Mandarin (*C. reticulata*) together with the grapefruit (*C. maxima*) and citron (*C. medica*) are three basic species of subgenus *Citrus*. Traditionally, morphological characters have been used to identify and characterize *Citrus*. However, there is a high level of genetic variability which can sometimes make an accurate separation for each variety. Although, there is a large amount of variability within the *Citrus* genus with which the breeder can work and closely related genera provide a wider selection of characters. Morphological characterization in combination with molecular markers would be more rewarding in terms of accurate identification and characterization of most closely related cultivars at intra-specific level. Presently, molecular marker techniques are routinely used for proper characterization, management and conservation of germplasm collections of horticultural species [15].

Several molecular markers viz. random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeats (SSRs), amplified fragment

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length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), coding and non-coding regions of chloroplast DNA, internal transcribed spacer (ITS) region, etc. have been used for analysis of genetic diversity and relationships, cultivars identification, linkage mapping, and molecular phylogeny of *Citrus* [6, 12, 14, 16, 19].

Among the molecular markers, RAPD and ISSR markers have been extensively used to study genetic diversity and relationships in *Citrus* species [1, 2, 4–7, 9–11, 17, 21–23]. These markers can detect polymorphism in a single reaction with a good repeatability and reproducibility. The main objective of this study was to characterize mandarin cultivars using morphological and molecular markers, to evaluate the genetic diversity and relationships among 25 accessions of *C. reticulata*, collected from three regions of India, i.e., North, Central and North-Eastern.

Materials and Methods

Plant Materials

Leaves of 25 accessions of *C. reticulata* and its hybrid representing native as well exotic cultivars collected from three regions of India, i.e., 22 accessions from Punjab Agricultural University (PAU), Regional Station, Abohar (North region), 1 accession from National Research Centre on Citrus (NRCC), Nagpur (Central region) and 2 accessions from North-Eastern states, were considered for the present study (Table 1). Leaf material from each accession was also taken for Herbarium collection along with morphological characterization.

Morphological Analysis

Eleven quantitative and 22 qualitative characters were evaluated using leaves, fruits and seeds characters of 25 accessions under study. The selection of morphological characters was made based on the descriptors developed by International Plant Genetic Resources Institute (IPGRI), now Bioversity International [13]. Morphological character data were standardized using the YBAR option of the Stand program from the NTSYS-pc 2.1 software [20]. Duplicate measurements for each specimen were averaged and used to design a data matrix of pairwise similarities between genotypes. The simple matching coefficient (SMC) was used to measure similarity, as it was the coefficient with the best results following a cophenetic test. Principal components analysis (PCA) was used to depict non-hierarchical relationships among the cultivars. Eigenvalues and eigenvectors were calculated by the Eigen program using a correlation matrix as input (calculated using standardized morphological data) and 2D plot was

used to generate the two-dimensional PCA plot from the software NTSYS-pc 2.1 [20].

Genomic DNA Extraction and Quantification

Total genomic DNA was isolated from 25 accessions using cetyl trimethyl ammonium bromide (CTAB) extraction protocol [8] and then quantified spectrophotometrically on ND1000 UV-spectrophotometer (Thermofisher Scientific Pvt. Ltd.).

RAPD-PCR Amplification

Fifty decamer primers (Operon Technologies Inc.) were screened in mandarin cultivars, of which 15 primers generated polymorphic and reproducible banding patterns and were selected for final analysis. PCR amplification was carried out in 25 µl reaction volume containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.0–2.5 mM MgCl₂, 0.2 mM dNTP each, 1.0 U Taq DNA polymerase (Bangalore Genie, India), 10 pM primer and 25–30 ng genomic DNA. The amplification was performed in a PTC–200 Thermocycler (MJ Research, Massachusetts, USA), with reaction conditions programmed as initial pre-denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing 37 °C for 1 min, and extension at 72 °C for 2 min. A final extension was given for 7 min at 72 °C. Amplification products were separated by electrophoresis on 1.5 % Agarose gels stained with ethidium bromide at 100 V for 3 h and bands were visualized and documented in AlphaImager® HP (Alpha Innotech Corporation).

ISSR-PCR Amplification

A total of 100 primers identified by the University of British Columbia (UBC) were procured from Geno Biosciences Pvt. Ltd. and used for ISSR-PCR optimization trials. Eleven primers, which gave the best amplification results with the sample DNA, were selected for final ISSR-PCR analysis. PCR-amplification was carried out in 25 µl reaction volume containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.0–2.5 mM MgCl₂, 0.2 mM dNTP each, 1.0 U Taq DNA polymerase (Bangalore Genie, India), 0.2 µM primer and 25–30 ng genomic DNA. The amplification was performed in a PTC–200 Thermocycler (MJ Research, Massachusetts, USA), with reaction conditions programmed as initial pre-denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. A final 7 min extension at 72 °C followed the completion of 35 cycles. Amplification products were separated by electrophoresis on 1.5 % Agarose gel stained with ethidium bromide at 100 V

Table 1 Parentage status of studied Mandarin accessions collected from Abohar (PAU, RS- Punjab Agriculture University, Regional Station), Khasi Hills (NEH, Meghalaya), Nagpur (NRCC- National Research Centre for Citrus) and Sikkim

S. no	Citrus cultivar	Scientific name	Parentage	Origin	Collection site
1	Laddu	<i>C. reticulata</i>	Natural selection	India	Abohar
2	SZ-In-com	<i>C. reticulata</i>	Natural selection	USA	Abohar
3	Willow leaf	<i>C. reticulata</i> <i>C. deliciosa</i> Ten.	Parents unknown	Unknown	Abohar
4	Butwal	<i>C. reticulata</i>	Natural selection	India	Abohar
5	Hazara	<i>C. reticulata</i>	Natural selection	India	Abohar
6	Srinagar	<i>C. reticulata</i>	Natural selection	India	Abohar
7	Clementine	<i>C. clementina</i> hort. ex Tanaka	Mandarin × sour orange	Algeria	Abohar
8	Cleopatra	<i>C. reshni</i> hort. ex Tanaka	Open pollinated seedling	India	Abohar
9	Kinnow (<i>Citrus</i> × <i>nobilis</i>) × (<i>Citrus</i> × <i>deliciosa</i>)	<i>C. reticulata</i>	King tangor × willow leaf mandarin	USA	Abohar
10	Orlando	<i>C. reticulata</i>	Duncan grapefruit × dancy tangerine	Florida USA	Abohar
11	King	<i>C. nobilis</i> Loureio	Natural tangor	India	Abohar
12	Temple	<i>C. reticulata</i> <i>C. temple</i> Hort. Ex Y. Tanaka	Natural selection	Jamaica	Abohar
13	Fairchild	<i>C. reticulata</i>	Clementine mandarin × orlando tangelo	USA	Abohar
14	Fremont	<i>C. reticulata</i>	Clementine × ponkan	Florida, USA	Abohar
15	Orange East Indian	<i>C. reticulata</i>	Natural selection	India	Abohar
16	Nasnaran	<i>C. amblycarpa</i> (Hassk.) Ochse	Natural selection	Java, Indonesia	Abohar
17	Wilking	<i>C. reticulata</i>	Willow Leaf mandarin × king tangor	California, USA	Abohar
18	Dweet tangor	<i>C. reticulata</i>	Mediterranean sweet orange × dancy tangerine	California, USA	Abohar
19	Dancy	<i>C. reticulata</i> <i>C. tangerina</i> Tanaka	Natural selection	Morocco	Abohar
20	Fewtrell's early	<i>C. reticulata</i>	Clementine mandarin × orlando tangelo	California, USA	Abohar
21	Ponkan	<i>C. reticulata</i>	Natural selection	China	Abohar
22	Minneola	<i>C. reticulata</i>	Duncan grapefruit × dancy mandarin	Florida, USA	Abohar
23	Nagpur mandarin	<i>C. reticulata</i>	Natural selection	India	Nagpur
24	Khasi mandarin	<i>C. reticulata</i>	Natural selection	India	Meghalaya
25	Sikkim mandarin	<i>C. reticulata</i>	Natural selection	India	Sikkim

for 3 h, and bands were visualized and documented in AlphaImager[®] HP (Alpha Innotech Corporation).

Data Analysis

Amplified bands generated from RAPD and ISSR-PCR amplification were scored based on the presence (1) or absence (0) of bands for each primer and used to calculate a genetic similarity matrix employing the SMC using software NTSYS-pc version 2.1 [20]. Cluster analysis was performed on both morphological and molecular data using the “unweighted pair group method using arithmetic means”

(UPGMA) algorithm, from which dendrograms depicting similarity among varieties were drawn and plotted using NTSYS-pc software. The cophenetic correlation was calculated to find the degree of association between the original similarity matrix and the tree matrix in both morphological and molecular analysis. Comparison between both the methods was performed for the accessions for which both data were available by calculating the correlation between the two data sets using the Mantel test [18] in NTSYS-pc. Principal component analysis (PCA) was also carried out to find out genetic association among the cultivars using same software.

Results

Morphological Analysis

Comparative analysis of 33 morphological characters of *C. reticulata* accessions collected from different states of India revealed moderate variation. A pair-wise similarity among the accessions of *C. reticulata* ranged from 0.15 to 0.73 with an average of 0.44 based on morpho-metric data. Maximum similarity (0.73) was observed between Fewtrell's Early and Dweet Tangor cultivars, while minimum (0.15) was observed between Butwal and Willow Leaf, King and Willow Leaf, King and Srinagar, Fair Child and Butwal, Fremont and Butwal, Ponkan and King, Sikkim Mandarin and Butwal (Fig. 1) cultivars.

A dendrogram was generated based on morpho-metric data in which three major clusters were formed for the 25 accessions under study (Fig. 1). The first cluster comprised of six cultivars, namely, Laddu, Orange East India, Cleopatra, Butwal, Hazara and Nagpur mandarin. In this cluster, Nagpur

Fig. 1 Dendrogram generated based on morphological traits of 25 cultivars of *C. reticulata* using UPGMA method

mandarin was the most distinct cultivar among all the other cultivars of this cluster and separated with a similarity value of 0.39. Butwal and Hazara were highly similar morphologically showing a similarity value of 0.60. The second cluster was the biggest one comprising of 18 cultivars, viz. SZ-In-Com, Ponkan, Clementine, Temple, Wilking, Willow Leaf, Khasi mandarin, Sikkim mandarin, Nasnaran, Dancy, Srinagar, Kinnow, Fair child, Orlando, Fremont, Dweet tangor, Fewtrell's early and Minneola. Within this cluster, the cultivars Dweet tangor and Fewtrell's Early were highly similar morphologically showing a similarity of 0.73. The third cluster comprised of only one cultivar viz. King which was morphologically very distinct from remaining 24 cultivars having a similarity value of 0.25.

Based on Mantel Z-statistics [18] the correlation coefficient (r) was estimated at 0.89. A value of 0.89 is considered as a good fit of the UPGMA cluster pattern to the data. Two-dimensional plot (2D plot) generated from PCA showed three groups which was found almost similar to the clustering pattern of UPGMA dendrogram. In 2D plot, the cultivar Fremont constituted one group whereas in UPGMA clustering, Fremont was grouped together with 17 other cultivars in one cluster. SZ-In-Com and Kinnow also formed separate group in 2D plot but in the dendrogram it was grouped along with Fremont. King and Wilking together constituted one group in 2D plot, whereas in the dendrogram, they were present in two different clusters and King was distinct from other cultivars in UPGMA dendrogram. The analysis gave 25 principal components out of which first ten principal components contributed 82.85 % of the total variability. The first five principal components accounted for 55.44 % of the total variation and the first three accounted for 38.09 % of the variation, in which maximum variation was contributed by first component (14.79 %) followed by second component (12.47 %), and third component (10.83 %). The first PC was highly influenced by characteristics of the fruit morphology viz. shape of fruit base, seed colour, leaf lamina shape, oil gland size, density of oil gland and petiole wing shape (Table 2). In second PC, the traits contributing to the total variability were fruit adherence of albedo to pulp, fruit length, seed colour, oil gland size, nature of oil gland and leaf apex. The third PC was influenced by cotyledon colour, petiole wing width, adherence of albedo to pulp, pulp firmness, seed surface and total soluble sugars (TSS) content.

RAPD Analysis

Fifteen primers were selected for the RAPD analysis based on the reproducibility and banding patterns. A total of 217 bands were generated from 15 RAPD primers, of

Table 2 Eigenvectors of morphological variables explained by first three principal components

S. no.	Characters	PC-1	PC-2	PC-3
1	Fruit shape	0.0501	0.1637	0.2619
2	Shape of fruit base	0.3485	−0.626	−0.124
3	Shape of fruit apex	0.3412	−0.4215	−0.2805
4	Fruit skin colour	−0.1538	−0.5434	0.2992
5	Fruit surface texture	0.0608	−0.1438	0.2416
6	Adherence of albedo to pulp	0.052	0.6312	0.394
7	Nature of oil glands	0.2659	0.4543	−0.0735
8	Density of oil glands on fruit surface	0.4469	0.3378	−0.2987
9	Oil gland size on fruit surface	0.5352	0.4578	0.2074
10	Albedo colour	−0.2253	0.279	0.0855
11	Pulp colour	−0.1409	−0.4286	−0.099
12	Pulp firmnes	0.2396	−0.3017	0.3357
13	Seed shape	0.1501	−0.2791	0.1308
14	Seed surface	0.1432	−0.2635	0.3032
15	Seed colour	0.6996	0.4801	0.2302
16	Colour of cotyledons	−0.0515	−0.1723	0.519
17	Chalazal spot colour	−0.515	−0.2895	−0.2913
18	Fruit weight (g)	−0.2167	0.2598	−0.4591
19	Fruit diameter (cm)	−0.8224	0.209	0.0456
20	Fruit length	−0.5678	0.3428	0.0792
21	Width of epicarp at equatorial area (mm)	−0.5732	0.2528	−0.456
22	Fruit rind thickness	−0.7429	−0.0953	0.0445
23	No. of segments per fruit	0.2186	0.2267	−0.3267
24	Juice content in endocarp	−0.3583	−0.0453	0.3134
25	Taste of juice TSS	−0.2639	−0.03	0.3797
26	No. of seed/fruit	−0.2854	−0.2324	0.0399
27	10 Seed wt. (gm)	−0.1239	−0.0482	−0.445
28	Leaf lamina shape	0.5336	−0.1657	−0.4444
29	Leaf lamina margin	0.2288	0.0249	−0.5647
30	Leaf apex	−0.1152	0.448	−0.3274
31	Abvsence/presence of petiol wings	0.1404	−0.276	−0.0026
32	Petiol wing width	0.325	0.1704	0.5076
33	Petiole wing shape	0.3768	−0.3756	−0.2255

which 207 bands were polymorphic (95.37 %) with an average of 13.8 polymorphic bands per primer. The fragment size ranged from 0.3 to 5 Kb (Table 3). A representative gel profile generated using primers OPA-08 is shown in Fig. 2a. Each primer amplified at a range of 4–19 amplicons with an average of 14.47 amplicons per primer. OPA-07 and OPA-13 primers amplified the maximum number of 19 amplicons, whereas OPA-06 primer generated the minimum number of 4 amplicons with 100 % polymorphism. The RAPD primer OPA-08 resulted in 18 amplicons of size range between 0.3 and 3.5 kb with PIC value 0.520 and one distinct band which distinguishes Sz-In-com from other selected accessions. The polymorphism percentage ranged from 78.57 (primer OPE-20) to 100 % (OPA-01, OPA-04, OPA-06, OPA-09,

OPA-11, OPA-13 and OPE-03). Average polymorphism across all the 25 cultivars was 95.37 %.

Four RAPD primers amplified four unique bands in specific mandarin cultivars. These primers amplified a specific DNA fragment which distinguished one cultivar from the others. Pattern of distribution of bands across all cultivars revealed that the primer OPA-08, OPA-10, OPA-17 and OPA-18 amplified a single fragment in SZ-In-Com (920 bp), Dancy (1000 bp), Fairchild (920 bp) and Fewtrell's Early (970 bp), respectively.

A maximum similarity value of 0.96 was observed between cultivars Hazara and Butwal, indicating that they were genetically most similar, whereas Fewtrell's Early and Srinagar; Fewtrell's Early and Orlando and Nagpur mandarin and Srinagar showed least similarity coefficient

Table 3 Details of amplified bands generated in 25 cultivars of *C. reticulata* based on 15 RAPD and 11 ISSR primers

S. no.	Primer	Sequences (5'–3')	Total no. of amplified bands	No. of polymorphic bands	% of polymorphism	Range	PIC value
RAPD							
1	OPA-01	CAGGCCCTTC	18	18	100.00	500–4,000	0.681
2	OPA-02	TGCCGAGCTG	14	13	92.85	375–4,000	0.690
3	OPA-04	AATGGGGCTG	17	17	100.00	500–3,000	0.678
4	OPA-06	GGTCCCTGAC	04	04	100.00	1,200–3,500	0.597
5	OPA-08	GTGACGTAGG	19	18	94.73	300–3,500	0.520
6	OPA-09	GGGTAACGCC	15	15	100.00	700–4,000	0.563
7	OPA-10	GTGATCGCAG	19	18	94.73	325–3,500	0.537
8	OPA-11	CAAATCGCCGT	16	16	100.00	490–3,500	0.589
9	OPA-13	CAGCACCCAC	19	19	100.00	550–2,500	0.694
10	OPA-17	GACCGCTTGT	13	11	84.61	500–5,000	0.439
11	OPA-18	AGGTGACCGT	14	13	92.85	400–3,500	0.779
12	OPC-02	GTGAGGCGTC	11	9	81.81	600–2,500	0.796
13	OPE-03	CCAGATGCAC	14	14	100.00	400–2,500	0.725
14	OPE-04	GTGACATGCC	13	11	84.61	520–2,500	0.814
15	OPE-20	AACGGTGACC	14	11	78.57	625–4,000	0.818
		Total	217	207	95.39		
ISSR							
1	UBC-807	AGAGAGAGAGAGAGAGT	21	20	95.23	300–2,500	0.704
2	UBC-808	AGAGAGAGAGAGAGAGG	18	17	94.44	300–1,500	0.659
3	UBC-810	GAGAGAGAGAGAGAGAT	14	14	100.00	250–1,500	0.627
4	UBC-811	GAGAGAGAGAGAGAGAC	12	12	100.00	400–2,000	0.690
5	UBC-812	GAGAGAGAGAGAGAGAA	11	10	90.90	400–1,400	0.618
6	UBC-825	ACACACACACACACACT	11	10	90.90	500–1,500	0.649
7	UBC-827	ACACACACACACACACG	10	10	100.00	350–950	0.695
8	UBC-841	GAGAGAGAGAGAGAGAYC	23	22	95.65	200–2,000	0.664
9	UBC-855	TGTGTGTGTGTGTGTGRT	19	19	100.00	300–2,000	0.598
10	UBC-858	ACACACACACACACACYT	08	08	100.00	480–950	0.722
11	UBC-864	ATGATGATGATGATGATG	13	11	84.61	480–1,500	0.679
		Total	160	153	95.62		

of 0.46. Average similarity value across all the cultivars was 0.71.

A dendrogram generated based on UPGMA method grouped all the 25 accessions into four major clusters. First cluster was the largest one comprising 17 accessions, viz. Laddu, Hazara, Butwal, Orange East India, Clementine, Cleopatra, Nasaran, Kinnow, Dancy, Wilking, Dweet Tangor, Willow Leaf, Srinagar, Temple, Fremont, Orlando and King. Within this cluster, the cultivars Butwal and Hazara showed 96 % genetic similarity, while Willow Leaf was distinct from other cultivars in this cluster with similarity coefficient of 0.69. Second cluster comprised of two cultivars naming SZ-In-Com and Fairchild which were closely related with similarity value of 0.81. Third cluster comprised of two cultivars viz. Khasi mandarin and Sikkim mandarin which were closely related with similarity value of 0.72. Fourth cluster consisted of four cultivars, i.e.,

Fewtrell's early, Ponkan, Minneola and Nagpur mandarin. Within this cluster, Ponkan and Minneola were closely related with similarity of 0.84 while Nagpur mandarin was distinct from other cultivars with similarity value of 0.59.

Based on Mantel Z-statistics [18], the correlation coefficient (r) was estimated to be 0.82. 2D plot generated from PCA of RAPD data was also in coherence with the clustering pattern of UPGMA dendrogram, except Nagpur mandarin, which was distinctly separated in the 2D plot, while in the dendrogram; it was grouped in cluster IV. First and second principal components accounted for 14.39 and 9.85 %, respectively, of the total variation.

ISSR Analysis

Eleven primers were selected for the ISSR analysis based on the reproducibility and banding patterns. A total of 160

Fig. 2 Representative gel profiles of *C. reticulata* based on RAPD and ISSR primers; **a** OPA-08 and **b** UBC-812, respectively showing unique band (*circled bands* are distinguishing the respective accession from other selected accessions)

bands were generated, of which 153 bands were polymorphic (95.62 %). Each primer amplified 8–22 polymorphic amplicons with an average of 13.9 amplicons per primer. UBC-841 primer amplified the maximum number of 22 amplicons, whereas UBC-858 amplified the lowest number of polymorphic amplicons 8. Figure 2b showed representative ISSR profile generated by primer UBC-812. The polymorphism percentage ranged from 84.61 (primer UBC-864) to 100 % (UBC-810, UBC-811, UBC-827, UBC-855 and UBC-858). Average polymorphism across all the 25 accessions was 95.59 %. Overall size of the PCR amplified fragments ranged from 200 to 2,500 bp (Table 2). Pattern of distribution of bands across all accessions of *C. reticulata* revealed that the primer UBC-812 (1,400 bp) and UBC-841(990 bp) for Dweet Tangor (Fig. 2b) and UBC-825 (1,500 bp) for Feutrell's Early, respectively, amplified a unique DNA fragment which distinguished one cultivar from the others.

A pairwise Jaccard's similarity co-efficient among all the 25 accessions of *C. reticulata* ranged from 0.42 to 0.96.

The maximum similarity of 0.96 was observed between cultivars Hazara and Butwal, indicating that they are genetically most similar, whereas Khasi mandarin and Nasran showed least similarity coefficient of 0.42. Average similarity across all the cultivars was 0.69. In the dendrogram all the 25 cultivars were grouped into three major clusters. First cluster was the biggest comprising of the 19 cultivars viz. Laddu, Hazara, Butwal, Kinnow, Cleopatra, Srinagar, Clementine, Orange East India, Wilking, Orlando, Temple, Fremont, King, Willow Leaf, Dweet Tangor, Dancy, Nagpur mandarin, Ponkan and Minneola, in which Butwal and Hazara were genetically most similar showing 96 % similarity, while Nagpur mandarin was diverse from other cultivars of this cluster with similarity value of 0.62. The second cluster comprised of four cultivars, i.e., Fewtrell's Early, SZ-In-Com, Fairchild and Nasran in which SZ-In-Com and Fairchild were showing 75 % genetic similarity, while Fewtrell's Early was most distinct from other cultivars with similarity of 0.59. Third cluster comprised of two cultivars viz. Khasi

mandarin and Sikkim mandarin which were closely related with similarity value of 0.67.

Based on Mantel Z-statistics [18], the correlation coefficient (r) was estimated to be 0.93. 2D plot generated from PCA of ISSR data also in coherence with the clustering pattern of UPGMA dendrogram, except Fewtrell's Early, which was grouped with Ponkan, Minneola and Nagpur mandarin in the 2D plot, while in the dendrogram; it was grouped in cluster II. First and second PCs accounted for 14.39 and 9.85 %, respectively, of the total variation.

Cumulative Data Analysis of Morphology, RAPD and ISSR

A pairwise similarity among the cultivars of *C. reticulata* was ranged from 0.46 to 0.93 with an average of 0.69 based on combined morpho-metric data, RAPD and ISSR data. Maximum similarity (93 %) was observed between Butwal and Hazara cultivars, while minimum was observed between Fewtrell's Early and Srinagar cultivars with the similarity value of 0.46.

A dendrogram generated based on combined morphometric data, RAPD and ISSR data grouped all the 25 cultivars into five major clusters (Fig. 3). First cluster comprised of seven cultivars namely, Laddu, Butwal, Hazara, Orange East India, Cleopatra, Kinnow and Wilking. Within this cluster Butwal and Hazara were most similar combined morphologically and genetically showing a similarity value of 0.93. In this group, Kinnow and Wilking were most similar to each other and distinct to other related cultivars of this cluster with similarity value of 0.73. Second cluster was the biggest one comprising of nine cultivars, viz. Srinagar, Clementine, Orlando, Temple, Fremont, King, Willow Leaf, Dweet Tangor and Minneola. Within this cluster, Temple and Fremont were most similar with similarity value of 0.81, while Willow Leaf was most distinct from all other cultivars of this group with similarity value of 0.63. Third cluster comprised of three cultivars, viz. SZ-In-Com, Fairchild and Nasnaran. In this cluster, SZ-In-Com and Fairchild showed 74 % similarity to each other, while Nasnaran was diverse with the similarity value of 0.64. Fourth cluster comprised of four cultivars namely, Dancy, Ponkan, Fewtrell's Early and Nagpur mandarin. In this group, Dancy and Ponkan found to be most similar with 64 % similarity, while Nagpur mandarin was most distinct to all other cultivars with similarity value of 0.57. Fifth cluster comprised of two cultivars, viz. Khasi mandarin and Sikkim mandarin showing 69 % similarity.

2D plot generated from PCA of combined morphometric data, RAPD and ISSR data (Fig. 4) also supported the clustering pattern of UPGMA dendrogram. In 2D plot, cultivar Fewtrell's Early alone constituted one group whereas in UPGMA clustering, Fewtrell's Early was

Fig. 3 UPGMA dendrogram of 25 cultivars of *C. reticulata* generated based on combined Morphometric, RAPD and ISSR data

grouped together with three other cultivars in one cluster. Nagpur mandarin and Nasnaran together constitute one group in 2D plot, whereas in the dendrogram they were present in two different clusters and Nagpur mandarin was distinct from each other cultivars in UPGMA dendrogram. The analysis gave 25 PCs out of which first ten PCs contributed 67.92 % of the total variability of the analyzed accessions. The first five PCs accounted for 44.04 % of the total variability and the first three accounted for 30.02 % of the variance, in which maximum variability was contributed by first component (12.24 %) followed by second component (9.15 %), and third component (8.63 %).

Discussion

Morphological, RAPD and ISSR markers analysis among the mandarin cultivars were successfully employed to estimate genetic variability and to establish genetic

Fig. 4 2-D plot of 25 cultivars of *C. reticulata* generated based on PCA of combined Morphometric, RAPD and ISSR data

relationships and origin of indigenous and exotic cultivars. Morphological characterization based on 33 characters revealed significant diversity (56 %) in leaf, fruit and seed traits. RAPD and ISSR data generated from 25 cultivars of mandarin with 15 and 11 primers, respectively, were sufficient to provide inferences on genetic differentiation and relationships among the cultivars. RAPD and ISSR markers also showed high level of polymorphism (95 %). A high level of polymorphism (86 %) was also reported by Campos et al. [3] in mandarins based on AFLP markers. Although, Coletta Filho et al. [5] reported very narrow genetic base of mandarin group using RAPD marker and propose that the mandarin group is a single species *C. reticulata*. PIC values were also recorded high in both RAPD (0.661) and ISSR (0.664) markers, showing the efficiency of molecular marker used to detect polymorphism within the mandarin group.

Jaccard's genetic similarity value of RAPD and ISSR was found in the range of 0.46–0.96 (average 0.71) and 0.42–0.96 (average 0.69) suggesting moderate level of genetic diversity within the mandarin group. This inference was correspondingly congruent with the results (genetic similarity, 0.77) derived from RAPD marker analysis in 35 mandarin accessions by Coletta Filho et al. [5]. This moderate level of genetic diversity within the mandarin group may be due to free genetic exchange within the mandarin group as well as closely related species like

C. sinensis (sweet orange) and *C. paradisi* to form natural and artificial hybrids like tangor and tangelo, respectively. Further backcross of tangor and tangelo with cultivars of *C. reticulata* makes the mandarin group more diverse morphologically as well as genotypically.

In all the dendrograms (Morpho-metric, RAPD, ISSR and Combined), Butwal, Hazara, Laddu, Cleopatra and Orange East India cultivars of Indian origin were grouped together in the same cluster, showing close genetic relationship among the cultivars. These cultivars are the natural selection and chance seedling derived from *C. reticulata*.

Sikkim and Khasi mandarins found a place in the same cluster in RAPD, ISSR, morpho-metric and combined dendrograms suggesting high genetic similarity between both the cultivars. These are the natural cultivars of mandarin having similar morphology and adapted to higher altitude in particular climatic condition in north-east region of India. Kinnow and Wilking grouped together in the combined dendrogram as both cultivars originated from the same parents King and Willow Leaf. In the combined dendrogram, both Dweet tangor and Minneola tangelo formed a separate group in the cluster II as Dancy tangerine involved as one of the parent in the origin of both the cultivars. Resolution of combined dendrogram was comparatively higher than the separate morpho-metric, RAPD and ISSR dendrogram in term of genetic relationship and origin of these cultivars. However, grouping of all the 25

cultivars in the dendrogram was congruent with the morphology and genetic origin of cultivars.

Morphological characters like shape of fruit base, seed colour, leaf lamina shape, oil gland size, density of oil gland and petiole wing shape, fruit adherence of albedo to pulp, fruit length, seed colour, oil gland size, nature of oil gland and leaf apex, cotyledon colour, petiole wing width, adherence of albedo to pulp, pulp firmness, seed surface and TSS content represent maximum variability revealed by first three principal components were identified for developing minimal descriptor. This is the first study where morphological markers have been substantiated with RAPD and ISSR markers to distinguish the mandarin cultivars.

Conclusions

Both morphological and molecular markers showed a high degree of variation among the selected mandarin cultivars. The present study revealed that morphological and both molecular markers can be successfully utilized for inferring genetic diversity and genetic relationship of mandarin group. Similarity between Sikkim and Khasi mandarin, Kinnow and Wilking, and Dweet tangor and Minneola tangelo further confirmed the importance of these markers for distinguishing the mandarin cultivars based on environmental and genetic factors. Sikkim and Khasi mandarin are considered as two different cultivars due to morphological variation, but it is confirmed using molecular markers that both are genetically similar and the variations are due to environmental and geographical factors as the Sikkim mandarin is cultivated on higher altitudes as compared to Khasi hills where Khasi mandarin cultivation is in practice. Results derived from this study would be useful for Citrus breeding programmes and also improvement of Citrus industry.

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