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Morphogenetic characterization of seeded and seedless varieties of Kinnow Mandarin (*Citrus reticulata* Blanco)

Sadaf Altaf[1], Muhammad Mumtaz Khan[2]*, Muhammad Jaffar Jaskani[3], Iqrar Ahmad Khan[4], Muhammad Usman[4], Bushra Sadia[5], Faisal Saeed Awan[5], Asif Ali[6], Azeem Iqbal Khan[7]

[1]Citrus Nursery Sanitation Laboratory, Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan
[2]Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, PO Box 34, Al-khod 123, Sultanate of Oman
[3]Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan
[4]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan
[5]Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan
[6]Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan
[7]Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan

***Corresponding author: mumtaz@squ.edu.om**

Abstract

Kinnow (*Citrus reticulata* Blanco) mandarin is a superior fruit crop in Pakistan; however, it is characterized by high number of seeds which is disliked by consumers and the processing industry. The objective of this work was to document the morphological and genetic differences in selected seeded and seedless Kinnow strains from two different locations i.e., Citrus Research Institute Sargodha and University of Agriculture, Faisalabad. The morphological characters suggested variations in external and internal characters of seeded and seedless fruits such as growth habit, fruit shape and texture. Likewise chemical composition of seedless fruit was different than seeded including TSS and Ascorbic acid. Whereas PCA analysis suggested fruit weight, peel weight, non-reducing sugars and fruit diameter were largely contributing for the variations among the seeded and seedless strains of Faisalabad and Sargodha regions. The genetic characterization was carried out using Random Amplification of Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) markers. Two unique fragments of sizes 1000 bp each were detected by RAPD primers GL A-2 and GL C-19 in seedless strains which can be used as a fingerprint for the identification of seedless plants. The results obtained from SSR analysis showed 81.62% of overall polymorphism in seeded and seedless strains of two locations. This variation will help to develop molecular markers for seedless Kinnow strains.

Keywords: Microsatellites; Citrus; Seedlessness, Molecular Markers; Genetic Characterization.
Abbreviations: AFLP_Amplified fragment length polymorphism; CTAB_Cetyl trimethylammonium bromide; QTLs_Quantitative trait loci; RAPD_Random amplified polymorphic DNA; RFLP_Restriction fragment length polymorphism.

Introduction

Kinnow is a cross between two Citrus cultivars King and Willow leaf and is named as Kinnow mandarin (Frost, 1935). Mandarins (Citrus spp.) are the second most important group of citrus plants in the world with the highest climatic adaptation among the cultivated citrus. Pakistan is considered as the sixth largest mandarin producer in the world with an estimated production of 103.410 hectograms on 196.500 hectares (FAOSTAT, 2012). In Pakistan, Sargodha and Faisalabad presents favorable climatic conditions for citriculture. The ecozones of the central and southern Punjab province are very conducive for the optimum quality production of Kinnow (Khalil et al., 2011). This loose skinned citrus has certain demerits of delayed ripening, alternate bearing and commercially unfavorable number of seeds per fruit (Khan et al., 1992; 1996) and high limonin content is a problem for processing industry. Seedlessness, regular fruit bearing, dwarf tree size, disease resistance and limonin free cultivars are the targets of many citrus breeding programs in the world. High number of seeds per fruit in Kinnow is the only constraint which limits its export beyond a certain level (Khalil et al., 2011). In Citrus, seedlessness is positively associated with triploidy (Jaskani et al., 2005; Usman et al., 2008). Other techniques for seedlessness involve mutation breeding, cytoplasmic male sterility, somatic hybridization, gene transformation and parthenocarpy. During the past few decades, a great deal of progress has been made in citrus somatic hybridization. More than 12,000 triploid citrus somatic hybrids have been produced from interploidal crosses (Grosser and Gmitter, 2011). Parthenocarpy is an economically valuable trait in a number of horticultural crops. Consumers often prefer seedless fruit for aesthetic reasons, because many such fruit have more attractive appearance and offer added convenience, in terms of preparation and consumption (Vardi et al., 2008). Characters related to plants, flowers, fruits and leaves were used by several researchers to describe and

characterize distinct mandarin varieties and its hybrids (Domingues et al., 1999; Sutarto et al., 2009). The use of molecular markers has been a valuable and precise instrument to assist the genetic variability of citrus species. Techniques like RFLP (Restriction Fragment length polymorphism) and RAPD (Random Amplification of polymorphic DNA) have been used in germplasm characterization of various citrus species (Colettafilho et al., 1998, 2000; Fang et al., 1998; Federici et al., 1998; Nicolosi et al., 2000). Microsatellites are sequences which are considered perfect markers for genetic and physical genome mapping, detection and inequity of genotypes. In citrus, this marker has been used in studies of phylogenetic analysis and linkage (Kijas et al., 1995, 1997; Thomas et al., 1998). The objectives of the present study were characterization of seeded and seedless Kinnow plants present in the germplasm pool at the Citrus Research Institute Sargodha and University of Agriculture Faisalabad, Pakistan; evaluation of the morphological and genetic diversity and establishment of genetic relationships between them using microsatellite and RAPD markers.

Results

Morphological characterization

Both seeded and seedless strains showed variations in their morphological and genetic traits. Growth habit was classified as spreading in 83%, and branching density as medium 91% in both strains. The spines were absent in almost all strains. Most of the plants 75% presented color of leaf blade as medium. The leaf color variegation was absent in 66% plants of both strains. In case of fruit external characters, fruit shape was spheroid in all seedless strains and spheroid to obloid in all seeded strains. The shape of fruit base was truncate to convex in all seedless and truncate in all seeded strains. However shape of fruit apex was truncate to round in all seedless and truncate in seeded strains. Fruit skin color was orange in all strains. The fruit surface texture was rough to smooth in seedless and pitted in seeded strains. Nevertheless, 75% of plants showed strong fruit attachment to stalk, thickness of segment wall as thin and fruit axis as semi-hollow. The results for physical characters of seeded and seedless fruits were highly significant in Faisalabad seeded strains. Seedless fruits showed less fruit weight (97.0 -141.8) gm compared with seeded fruit (162.7-173.1) gm (Table-4). Similar trend was observed for fruit diameter amongst different strains. Faisalabad seeded and both Sargodha strains were more in fruit length (57.25-61.48) mm compared with seedless Faisalabad 51.53 mm. The results for fruit rind thickness of Faisalabad seeded and Sargodha seedless strains were (5.24mm) and (4.43mm) respectively. Maximum number of healthy seeds (23) was observed in seeded strains of both localities. The number of abortive seeds was higher (13.11) in Faisalabad seeded compared with Sargodha seeded strain (6.33). TSS was higher in both strains of Sargodha (11.35-11.67). The acidity percentage was almost similar in all strains. Vitamin C contents were higher in seeded strains and Faisalabad seeded strains showed maximum vitamin C content (31.8mg/100ml). The results were momentous in case of reducing sugar contents of seeded strains of Faisalabad (2.28) and Sargodha (3.15) and seedless strains of Sargodha (3.14). Both seeded and seedless strains of Sargodha showed maximum reducing sugars content. TSS was higher in both seeded and Seedless strains of Sargodha and similar was the case with reducing and total sugars. Principle Component Analysis (PCA) for the seeded and seedless citrus strains collected from two different regions showed variations among the four groups like Faisalabad seeded, Sargodha seeded, Faisalabad seedless and Sargodha seedless. Based on different morphological characters it was found that fruit weight, peel weight, non-reducing sugars and fruit diameter were largely contributing for the variations among seeded and seedless strains of Faisalabad and Sargodha kinnow. PCA plot based on 1st and 2nd component factors (94.18% and 69.95%) of molecular data analysis of fourteen kinnow plants showed great variation (Fig-1). Faisalabad seedless strains were found to be the most divergent from rest of the kinnow strains included in this study followed by Faisalabad seeded which was also present away from the Sargodha seeded and Sargodha seedless strains. The Sargodha seedless and Sargodha seedless strains also showed diversity among each other.

Molecular characterization using RAPD

For the assessment of genetic variation in seeded and seedless strains of Kinnow, fourteen strains including ten seeded (Fsd-Faisalabad 1,2,3,4,5,6,7,8,9,10) and four seedless (ORI 1-3, ORI 4-6, ORI 1-7, NIAB) showed genetic divergence between seeded and seedless strains (Fig-2). The 16 RAPD primers generated a total of 161 bands, with an average of about 10 bands per primer, out of which 106 were found polymorphic showing 65.83% overall polymorphism (Table-1). The number of polymorphic bands (NPB) scored for individual primers ranged from 4 to 11 with an average of 6.63 bands. Genetic divergence and relatedness of seeded and seedless strains were measured by using Unweighted Pair Group of Arithmetic Means (UPGMA) Analysis. The dendrogram showed the formation of six groups; among these all seedless showed a very distinct behavior than seeded strains. A unique RAPD fragment of approximately 1000 bp was detected with a primer GLA-2 in ORI (4-6) strain (Fig-3). A mother banding pattern was also observed in two seedless strains ORI (4-6) and ORI-7 with the primer GLC-19 (Fig-4).

Molecular characterization using SSR Marker

Total eight primer pairs flanking microsatellite loci in the Citrus genome were designed. The SSR markers were able to clearly distinguish between different citrus strains. PCR amplification showed that all primer pairs amplified the desired target microsatellite from Citrus genomic DNA. In fourteen strains including ten seeded (Faisalabad) and four seedless (three from Sargodha and one of NIAB), eight SSR primers generated a total of 17 loci with an average of about 2.12 loci per primer set. Among these, 15 out of 17 loci were polymorphic showing 88.23% of over all polymorphism. All primers were polymorphic in 14 strains except one primer TAA-1 which showed monomorphic loci. The average band frequencies observed for specific primers ranged from 0.5 to 1.0. In nine seeded strains of Sargodha these eight primers generated a total of 16 loci, with an average of about 2 loci per primer. The 12 out of 16 loci were polymorphic showing 75% of overall polymorphism. Among eight primers six primers were polymorphic and two primers TAA-27 and TAA-45 showed monomorphism. Number of bands produced per genotype ranged from 8 to 14. Cluster analysis of 14 strains including ten seeded and four seedless placed all strains into six distinct groups. The maximum similarity was observed between genotypes Fsd-8 and Fsd-10 (100%; Fig-5). While minimum similarity was observed between genotypes ORI-7 and Fsd-2 as well as NIAB and Fsd-2 (35.29%). In group A, Fsd-1 and Fsd-4 showed close similarity while in group B, Fsd-7 and ORI (1-3) showed very close relationship. In group C, three strains Fsd-6, Fsd-8 and Fsd-10 showed maximum similarity. The other two

Table 1. List of seeded and seedless kinnow strains.

Strains	Code	Description	Locations
1	Fsd	Seeded	Screen house, UAF
2	Fsd	Seeded	”
3	Fsd	Seeded	”
4	Fsd	Seeded	”
5	Fsd	Seeded	”
6	Fsd	Seeded	”
7	Fsd	Seeded	”
8	Fsd	Seeded	”
9	Fsd	Seeded	”
10	Fsd	Seeded	”
11	ORI	Seedless ORI (1-3)	Citrus Research Inst., Sargodha
12	ORI	Seedless ORI (4-6)	”
13	ORI	Seedless ORI (7)	”
14	NIAB	Seedless	Screen house, UAF

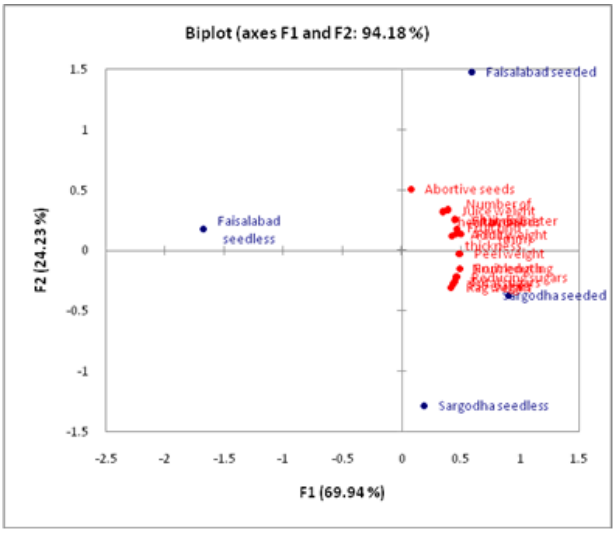

Fig 1. PCA biplot of seeded and seedless kinnow starins collected from two different locations i.e., Sargodha and Faisalabad.

strains Fsd-9 and NIAB seedless remained unclustered. In group D, two seedless strains, ORI (4-6) and ORI-7 showed maximum similarity. In group E, Fsd-5 strain remained unclustered showing a very distinct behavior from other strains. In group F, two strains Fsd-2 and Fsd-3 were clustered together.

Discussion

Evaluation of seeded and seedless strains showed similarities and variations in their characters. The similarities were observed in tree morphology of both strains. However, some genetic variations were accessed in case of seeded and seedless strains. These variations can be attributed to their genetic makeup (Bernier *et al.*, 1993), environmental conditions like temperature, humidity and rainfall (Pettersen *et al.*, 2006). The similarities were observed in scion trunk surface, tree shape, branch angle, vegetative life cycle, leaf division, leaf lamina shape, leaf lamina attachment, leaf apex and wings. This evidence was proved by Khan *et al.*, (1992) that leaf angle, length of leaf blade and petiole length did not differ significantly between genotypes. Similar was the case with adherence of albedo to pulp, nature and density of oil glands, albedo color, and areole, adherence of segments and segment shape uniformity, pulp and seed characters. In case of fruit weight seedless fruits showed less fruit weight compared with seeded fruit. The findings are similar to Gillaspy (1993) and Nitsch (1970) who stated that the developing embryos control the rate of cell division in the surrounding fruit tissues and the number of developing seeds influences the final size and weight of the fruit. The maximum peel thickness was observed in seedless strain. Variation in peel thickness of seedless Kinnow was also observed by Singh and Singh (2004) who reported maximum peel thickness 4.59 mm in seedless Kinnow and also reported that in Kinnow mandarin the peel weight and thickness were higher compared with other mandarins. Our findings are in accordance with these results because maximum thickness in case of seedless was 4.43 mm. Maximum number of healthy seeds was observed in seeded strains of both localities. The number of abortive seeds was higher in Faisalabad seeded compared with Sargodha seeded strain. Variability in fruit quality, the seed number per fruit and the number of abortive seeds was also studied previously (Altaf et al., 2008; 2009; Fatima et al., 2010). TSS of fruits was directly correlated with total and reducing sugar contents. It was higher in both seeded and seedless strains of Sargodha and similar was the case with reducing and total sugars. Possibly, it is due to soil conditions of two localities. The organic matter contents are higher in Sargodha soils ranging from 1 to 2% while in Faisalabad soils it ranges from 0.75 to 1.35% (Khan, 2008). The acidity percentage was almost similar in all strains. The results were in confirmation with Grewal et al. (2000) who reported that in Kinnow the acidity almost remains similar from February to April ranging from 0.51% to 0.71%. In case of RAPD marker, Group A and E contained all seeded strains

Table 2. RAPD primers with sequence, number of polymorphic bands (NPB) and mean band frequency (MBF).

PRIMER	SEQUENCE	NPB	MBF
GL DecamerA-02	TGCCGAGCTG	6	0.57
GL DecamerA-03	AGTCAGCCAC	7	0.78
GL DecamerA-04	AATCGGGCTG	4	0.68
GL DecamerA-05	AGGGGTCTTG	5	0.63
GL DecamerA-07	GAAACGGGTG	5	0.53
GL DecamerA-09	GGGTAACGCC	10	0.56
GL DecamerA-10	GTGATCGCAG	5	0.66
GL DecamerC-06	GAACGGACTC	7	0.58
GL DecamerC-15	GACGGATCAG	6	0.64
GL DecamerC-16	CACACTCCAG	9	0.46
GL DecamerC-19	GTTGCCAGCC	10	0.35
GL Decamer J-01	CCCGGCATAA	8	0.44
GL Decamer J-16	CTGCTTAGGG	4	0.65
GL Decamer K-4	CCGCCCAAAC	5	0.77
GL Decamer K-7	AGCGAGCAAG	11	0.72
GL Decamer K11	AATGCCCCAG	4	0.44

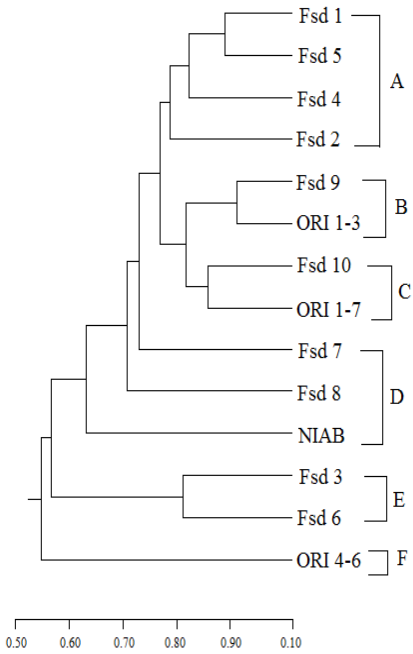

Fig 2. Dendrogram of fourteen seeded and seedless strains of Sargodha and Faisalabad obtained from similarity matrix based on Nei's UPGMA (RAPD).

Fsd 1, Fsd 5, Fsd 4, Fsd 2 and Fsd 3, Fsd 6 respectively. However group F contained only a seedless strain ORI 4-6. A single fragment of size 1000 bp (base pairs) was also detected in seedless strain by a primer GL Decamer A-2. The second mother banding pattern was observed by another GL Decamer C-19. This fragment can be further utilized for the development of Sequence Characterized Amplified Region (SCAR) markers to design a primer and can be used as a fingerprint for the identification of the seedless citrus plants. Whereas, other groups including B, C and D showed some correlation between seeded and seedless strains. The absence of genetic variability among these strains suggested that these are either clonal propagations or that these have undetectable genetic variability, such as point mutations which cannot be detected by RAPD (Colettafilho et al., 2000). Like RAPD, microsatellite also distinguished seedless from seeded and placed seedless into separate distinct groups. Group A, E and F comprised of all seeded strains Fsd-1, Fsd-4 and Fsd 5 and Fsd 2, Fsd 3 respectively. But group D comprised of two seedless strains ORI 4-6 and ORI-7 which were also distinguished by the presence of two unique bands by RAPD. Group B consisted of seedless strain ORI (1-3) and Fsd-7. In Group C, all seeded strains Fsd-6, Fsd-8, Fsd-9 and Fsd-10 showed close similarity but NIAB seedless remained unclustered. Presence of the genetic diversity among the seedy and seedless strains is due to the fact that all these strains are derived from some mutagenesis and SSR considered being the ideal technique to identify the single base alterations in the genomic regions. Same in this case as the data reported is based on only eight highly polymorphic primers and the results of the other primers gave monomorphic banding pattern (data not included). The similarity index depicted, there is less genetic difference among the seedless strains but if we compare the seedless and seedy germplasm, we found 40 to 60 % similarity among them. It means this similarity is only based on the 8 polymorphic primers. In conclusion, the seeded and seedless strains of Kinnow showed distinct morphological, physicochemical and genetic differences. The fruit chemical composition was relatively variable in both strains. On the other hand, the differences between morphological and molecular characters are apparently independent, due to diverse pressure and evolutionary factors. These diverse characters in this particular species may help in further breeding and crop improvement.

Materials and Methods

Plant material

A total of fourteen Kinnow plants were selected for evaluation including ten plants of seeded strains and four plants of seedless strains from germplasm pool of Orange Research Institute (ORI), Sargodha and University of Agriculture, Faisalabad, respectively (Table-1). These institutes are situated in Pakistan at latitude 31°15'N and longitude 73°03' E and 32°5' 1⅛ N and longitude 72°40' 16⅛ E, respectively. Both localities are approximately 94 km away from each other with subtropical climatic conditions. The maximum temperature of Faisalabad and Sargodha area in summer rises upto 48°C-50°C whereas minimum temperature in winter goes down to 4°C to freezing point, respectively. The average rainfall in both areas is ~40mm. All selected plants were of the same age, grafted on rough lemon rootstock and planted in a rectangular layout system.

Table 3. Details of SSR primers with amplified and polymorphic loci in fourteen seeded and seedless strains.

Sr. No.	Primer Name	Sequence (5'-3')	TNL	TML	TPL	MBF	AT
1	TAA1 F	GACAACATCAACAACAGCAAGAGC	1	1	0	1.0	58 °C
	TAA1 R	AAGAAGAAGAGCCCCCATTAGC					
2	TAA15 F	GAAAGGGTTACTTGACCAGGC	2	0	2	0.5	57 °C
	TAA15 R	CTTCCCAGCTGCACAAGC					
3	TAA27 F	GGATGAAAAATGCTCAAAATG	2	0	2	0.5	55 °C
	TAA27 R	TAGTACCCACAGGGAAGAGAGC					
4	TAA33 F	GGTACTGATAGTACTGCGGCG	2	0	2	0.5	58 °C
	TAA33R	GCTAATCGCTACGTCTTCGC					
5	TAA41 F	AGGTCTACATTGGCATTGTC	2	0	2	0.5	52 °C
	TAA41 R	ACATGCAGTGCTATAATGAATG					
6	TAA45 F	GCACCTTTTATACCTGACTCGG	2	0	2	0.5	56 °C
	TAA45 R	TTCAGCATTTGAGTTGGTTACG					
7	TAA52 F	GATCTTGACTGAACTTAAAG	3	0	3	0.38	47 °C
	TAA52 R	ATGTATTGTGTTGATAACG					
8	CAC23 F	ATCACAATTACTAGCAGCGCC	3	0	3	0.66	54 °C
	CAC23 R	TTGCCATTGTAGCATGTTGG					

TNL=Total no of Loci, TML= Total Monoporphic loci, TPL= Total Polymorphic Loci, MBF= Mean band frequency, AT= Annealing temperature

Table 4. Physicochemical characters of seeded and seedless Kinnow strains.

Strains		Characters Fruit weight (g)	Fruit diameter (mm)	Fruit length (mm)	Fruit rind thickness (mm)	Peel weight (g)	Rag weight (g)	Juice weight (g)	Number of healthy seeds	Abortive seeds	TSS (Brix)	Acidity (%)	Vitamin C (100mg/ml)	Reducing sugars (%)	Non reducing sugars (%)	Total sugars (%)
Seeded	Faisalabad seeded	173.11a	74.52a	57.25ab	5.23a	36.33b	28.55ab	103.0a	23.22a	13.11a	9.84b	0.71a	31.83a	2.82a	4.81ab	7.66bc
	Sargodha Seeded	162.78ab	69.48b	61.48a	3.80b	47.0	34.97ab	76.22b	23.00a	6.33b	11.67a	0.72a	28.16ab	3.15a	5.42a	8.86a
Seedless	Faisalabad seedless	97.00c	59.60c	51.13b	2.23c	17.0c	21.67b	47.0c	4.00b	6.66b	8.43b	0.68a	20.23c	2.21b	4.03b	6.46c
	Sargodha seedless	141.83b	68.21b	58.56a	4.43ab	32.16b	36.50a	61.67bc	1.00b	1.66c	11.35a	0.69a	24.93bc	3.14a	5.22ab	8.64ab

*Means sharing similar letters in columns are non-significant at ($P \leq 0.05$)

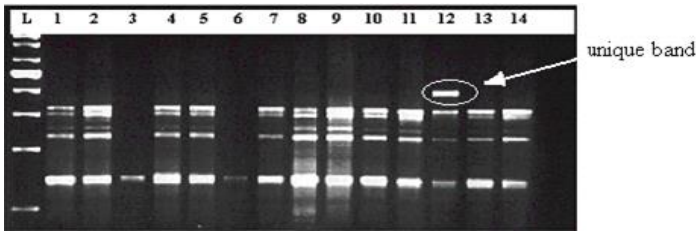

Fig 3. RAPD (PCR) of Seeded and Seedless strains with primer GLA-2. L is a 1Kb ladder and lanes include 1= Fsd-1, 2= Fsd-2, 3= Fsd-3,4 =Fsd-4, 5=Fsd-5, 6=Fsd-6, 7=Fsd-7, 8=Fsd8, 9=Fsd-9, 10=Fsd-10, 11=ORI-(1-3), 12=ORI-(4-6), 13= ORI-7, 14=NIAB.

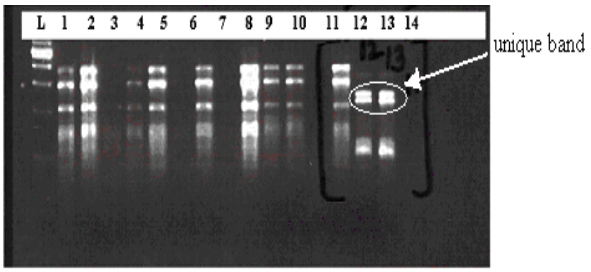

Fig 4. RAPD (PCR) of Seeded and Seedless strains with primer GLC-19. L is a 1Kb ladder and lanes include 1= Fsd-1, 2= Fsd-2, 3= Fsd-3,4 =Fsd-4, 5=Fsd-5, 6=Fsd-6, 7=Fsd-7, 8=Fsd8, 9=Fsd-9, 10=Fsd-10, 11=ORI-(1-3), 12=ORI-(4-6), 13= ORI-7, 14=NIAB.

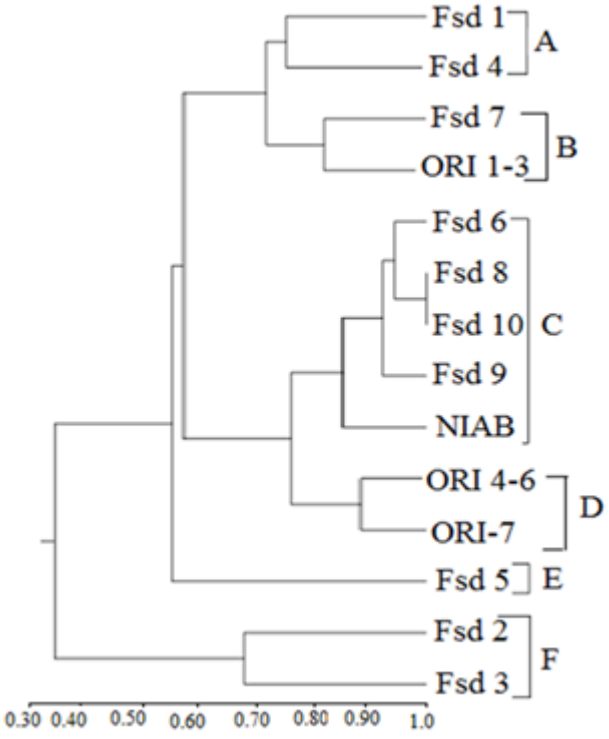

Fig 5. Dendrogram of fourteen seeded and seedless strains of Sargodha and Faisalabad obtained from similarity matrix based

The plants were grown in open field conditions. The seedless strains evaluated from Sargodha were selections and from NIAB (Faisalabad) were mutants developed through irradiations (Altaf *et al.,* 2004).

Morphological characterization

The morphological features of tree and leaves were evaluated in the field, whereas physico-chemical analysis of fruits was carried out in the laboratory. All morphological characterization was described following standard Citrus descriptors (IPGRI, 1999). Three ripe fruits from each plant were randomly evaluated and collected during harvesting season in February. The chemical composition was analyzed by measuring TSS, acidity, vitamin C, reducing, non-reducing and total sugars by the method described by (Lane and Eynon, 1923; Hortwitz, 1960; Ruck, 1961; Soule and Grierson, 1989).

Genomic DNA extraction

Total genomic DNA was isolated from fully expanded leaves using the CTAB (hexadecyltrimethylammonium-bromide) method (Khan *et al.,* 2004) with few modifications. Briefly, 2 g of leaves were ground in liquid nitrogen to a fine powder. Extraction was done by using cetyl trimethyl ammonium bromide (CTAB). The DNA was washed with 70% ethanol and dissolved in concentration was determined spectrophotometrically at 260 nm. Stock DNA samples were stored at –20 °C and diluted to 20ng and 50ng uL^{-1} for RAPD and SSR respectively.

RAPD analysis

All fourteen genotypes were analysed by 16 RAPD primers (Table-2) for PCR amplifications. The RAPD primers were purchased from Gene Link Company USA. The basic protocol for PCR was performed in a total volume of 25µl, containing 20ng of template DNA, 2.0µL of single primer, 0.2µl of Taq DNA polymerase, 0.20mM of each dNTPs, 2.5µl of 10x PCR buffer, 50 mM $MgC1_2$ and 0.025% of 2.5µl gelatin. DNA amplifications were carried out in a Thermal Cycler Eppendorf AG No. 533300839 (Germany). The PCR reaction conditions were established for 40 cycles of 94°C for 5 minutes of denaturation, 35°C for 2 minutes of annealing, 72°C for 2 minutes of elongation, final extension step at 72°C for 10 minutes. The amplification products were visualized in an ultraviolet transilluminator, after horizontal electrophoresis in 1.2% agarose gels, using the TBE 1x buffer, the gel was stained with ethidium bromide. A photographic record was taken under UV illuminations.

SSR analysis

Same genotypes were evaluated by 8 SSR primers (Table-3) from a previous study (Kijas *et al.,* 1997). The PCR reaction volume was 22.5µl consisting of 50ng of genomic DNA, 0.2µl of *Taq* DNA polymerase, 2.0µl of primer, 2.5µl of 10x PCR buffer, 0.2 mM of 4µl dNTPs and 50 mM of 3µl $MgC1_2$ and 8.3µl of sterile distilled water. The PCR thermal profile was 32 cycles of 94°C for 5 minutes of denaturation, 55°C for 30 seconds of Annealing, 72°C for 4 minutes of elongation and final extension step at 72°C for 10 minutes. The PCR products were separated by vertical electrophoresis in 40% of acryl amide gel, using a 100-bp DNA step ladder to allow consistent allele size calling for all loci and samples tested.

Statistical analysis

Chemical composition of fruit was analysed by completely ramdomized design (CRD) using least significant difference test. A principal component analysis (PCA) was done for all morphological characters using XLSTAT software. For molecular characterization only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using Popgene-32 software, version 1.44 (Yeh *et al.*, 2000). The dendrogram was constructed by using a distance matrix using the unweighed pair group method with arithmetic average (UPGMA) sub-program of NTSYS-PC to estimate genetic differences of seeded and seedless Kinnow strains.

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