

Molecular characterization of Kinnow mandarin clones and mutants using cross genera SSR markers

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Kinnow mandarin is a highly priced commercial citrus fruit for domestic as well as overseas markets. Excessive numbers of seeds in the fruit makes it less preferred mandarin for dessert as well as processing purposes. Attempts were made in the past to collect superior less seeded clones of Kinnow. Besides, efforts were also made to induce variation in Kinnow using physical (γ -ray) and chemical (ethyl methanesulphonate) mutagens. The non-availability of SSR markers in Kinnow made us look into cross genera marker polymorphism and characterization of these clones. The present study demonstrated the cross-genera transferability of simple sequence repeat (SSR) markers developed in citrus and chickpea to Kinnow mandarin clones and mutants. Out of 87 primers tested (67 from chick pea and 20 from citrus), 11 primers (8 citrus and 3 chickpea) showed polymorphism and amplified 14 alleles. Of 67 SSR loci (chick pea), 3 (4.47 %) gave cross-amplification. The polymorphic information content (PIC) value ranged 0.14 to 0.99, while Jaccard's similarity coefficients ranged 0 to 0.9. Clustering pattern showed that all the mutants and clones distinctly grouped and were different from the parent. It showed that mutants and clones had distinct molecular changes in them. In principle coordinate analysis, the first three coordinate axes accounted for 67.86% of the variation observed. Further, analysis of molecular variance (AMOVA) showed 80% genetic variation within the population and 20% among the population.

Keywords: Cross genera markers, Kinnow, molecular markers, mutants, mutation breeding

Introduction

Citrus is the third most important fruit crop in India after banana and mango¹. The most important commercial citrus group in India is mandarin (*Citrus reticulata* Blanco), followed by sweet orange (*Citrus sinensis* Osbeck) and acid lime (*Citrus aurantifolia* Swingle). Among mandarins, Kinnow mandarin (*Citrus nobilis* Lour $\times$ *Citrus deliciosa* Tenora), one of the introduced citrus varieties, occupies a predominant place in the Indian citrus industry. In the recent past, a rapid expansion in area under Kinnow has been observed in the States of Haryana, Punjab, adjoining areas of Delhi, Nagpur and Akola regions of Maharashtra because of its profuse bearing, high quality fruit and higher economic return. However, excessive seediness (25-35 seeds/fruit) has become the major cause for the annoyance of consumer preference and processing industries, where the less seeded sweet orange enjoys a monopoly. In fruit crops, clonal selections and induced mutants exhibit differential variation in morphological and

physico-chemical traits as compared to the parental genotype. The variation in agronomic traits observed in clonal selections and induced mutants can be epigenetic or genetically governed. The success of clonal selection and mutation as fruit breeding methods in perennial fruit trees is only of significance when positive mutation occurs and the same is genetic in nature. It is, therefore, important to ascertain that variations observed in clonal selections and mutants are genetically governed. The knowledge regarding genetic diversity, relatedness within and between clonal and mutant population of citrus, and their wild relatives are of paramount importance to ensure the long-term success of citrus improvement programmes. In this direction, molecular markers have been proved much efficient and powerful tools. These markers have greatly enhanced the efficiency of breeding several-fold, since selection is not directly based on the trait of interest but on the molecular marker tightly associated to the trait, thereby accelerating the generation of new varieties, especially when the characters are difficult to score².

Among the available molecular markers, microsatellites or simple sequence repeat (SSR) is used

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as an ideal tool in a variety of applications. Fairly well retention of repeat motifs supported the microsatellites to be useful tools for evaluating *Citrus* species and the related genera, thus reducing the complications faced in conventional breeding. However, for the development of SSR markers, the cost and effort required is one of the major constraints. Thus, a more extensive use of SSRs in plants would be facilitated if such loci were transferable across species even in closely related genera^{3,4}. So now, availability of large number of microsatellite markers in citrus as well as transferable cross genera SSR markers is offering immense scope in assessing the diversity and utilizing the diverse lines in map construction. They also provide new insights into genome analysis, help in germplasm characterization, phylogenetic analysis and genetic diagnostics. It is important to characterize the genetic diversity in plant species since they serve as a resource base for yet unidentified genetic information⁵.

Considering the above, an investigation was planned for molecular characterization of Kinnow clones and mutants developed from a pure Kinnow line maintained at the Division of Fruits and Horticultural Technology, IARI, New Delhi, India.

Materials and Methods

Experimental Material

In the present study, in total 13 clones and mutants were used for the purpose characterization. Leaf samples of all accessions were obtained from the orchard of Citrus Collection, Fruits and Horticultural Technology Division, IARI, New Delhi in the month of November, 2013.

DNA Isolation and PCR Analysis

DNA was isolated from young expanding leaves of clonal selections, mutants and parent using the modified CTAB method⁶. A total of 87 SSR primers (20 from citrus⁷⁻⁹ and 67 from chick pea^{10,11}) were used for studying the polymorphism among 4 clonal selections, 8 mutants and parental Kinnow. For PCR reactions, a 10 µL final volume containing, 20 ng of genomic DNA, 10 mM dNTPs, 200 pmole of each primer, 1 unit of *Taq* polymerase (Merck) and 1 µL *Taq* buffer (10×) was taken. The reactions were carried out in a Touchdown thermal cycler of Biorad MyCycler thermal cycler, USA and cycling parameters included 5 min of denaturing at 94°C, following 35 cycles of these steps: 1 min of denaturing at 94°C, 1 min of annealing temp 58°C, 2 min of elongation at 72°C, and lastly one cycle at 10 min at

72°C. Touchdown of 0.5°C for the first 20 cycles was carried out. Finally, the amplified fragments were resolved on 2% agarose gel and experiment was repeated twice.

Data Analysis

Number of polymorphic bands generated by each primer was scored as 0 or 1 for absence or presence of band, respectively. Each band in a band pattern of a specific microsatellites marker was recorded for each genotype and was assigned a letter. Alleles were numbered as 'a1', 'a2' etc. sequentially from the largest to the smallest sized band. No distinction was made between amplified products of varied intensity, when the amplified products were within the expected size range. Any marker with more than or equal to 30% missing data across various genotype was excluded from the analysis. Data was analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc version 2.1) software package¹². The similarity matrix was used to construct a dendrogram using the Unweighted Pair Group Method Arithmetic Average (UPGMA) to determine genetic relationships among the germplasm studied. Polymorphism Information Content (PIC) provides an estimate of the discriminatory power of a locus by taking into account not only the number of alleles that are expressed but also the relative frequencies of those alleles. PIC was calculated from the algorithm developed using all the primer combinations¹³. Marker Index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit¹⁴. Software GenAlEx V6.5¹⁵ was used for the Principle Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA).

Results and Discussion

The knowledge regarding genetic diversity, relatedness within and between clonal and mutant population of citrus, and their wild relatives are of paramount importance to ensure the long-term success of citrus improvement programmes. In this direction, molecular markers have proved as an efficient and powerful tools² for elucidating genetic diversity, determining parentage and revealing phylogenetic relationships among various *Citrus* species. However, accessions originated through spontaneous mutation are not easily distinguished¹⁶. Among the various DNA based markers, the microsatellite markers have become highly popular^{14,2}. The utility of SSR markers for a wide array of applications in genetics and breeding of

citrus has been well demonstrated by several researchers^{8,17,18}.

In the present investigation, 87 SSR primer pairs were deployed to study the polymorphism, of which only 20 generated bands. As 9 of the primers generated monomorphic bands, therefore only 11 polymorphic primers were deployed for molecular characterization of the clonal selections, mutants and parental Kinnow lines. These marker pairs gave 12.6% polymorphism. Since, there has been no prior report of available SSR markers in Kinnow, therefore these would serve as an important repertoire for future studies. These 11 SSR primer pairs amplified 14 amplicons in total, on an average 1.27 amplicons/primer pair (Table 1). The STMS primers, AMB1, CCSM147 and TS47 consistently amplified 2 bands as experiment was repeated twice. The reasons for occurrence of two bands deviating from the expected single band for a homozygous genotype could be residual

heterozygosity, generated due to mutations or variations at the molecular level in the somaclones¹⁹.

Of 67 SSR loci (chick pea), 3 (4.47 %) gave cross-amplification. These 3 primer pairs were found to be transferable to all 13 clones and mutants (Table 1). A more extensive use of SSRs in plants would be facilitated if such loci were transferable across species even in closely related genera^{3,4}. The PIC ranged 0.14 to 0.99 (Table 1). Kinnow being narrow in genetics base, most of the regions are highly conserved, which results in such a low polymorphism. But there was reasonably high rate of polymorphism for CCSM147 (2 alleles out of 11 STMS markers loci), indicating the scope for further utilization of this marker for characterization of Kinnow variants. The occurrence of unique alleles or rare STMS alleles provides an immense opportunity for generation of comprehensive fingerprint database.

The Jaccard's similarity matrix dendrogram constructed using the UPGMA method showed that

Table 1 — Properties of SSR polymorphic markers used for molecular characterization of Kinnow genotypes

Primer	Sequence (5'-3')	Allele size (bp)	PIC values	MI
AMB1 (Citrus)	F: TCGGCGTTCTCTCTCTCTCT R: TACTTGGACGTCGGGGACTA	100 (a1) 500 (a2)	0.92	1.84
AMB2 (Citrus)	F: ACGCATTTGACTGCTCTGTG R: AATTCAGAGGTGCCGTGAG	160 (a1)	0.14	0.14
CCSM13 (Citrus)	F – CTAGAGCCGAATTCACC R – AACAGCTACCAAGACACC	900 (a1)	0.97	0.97
CCSM18 (Citrus)	F- GTGATTGCTGGTGTCGTT R -AACAGTTGATGAAGAGGAAG	230 (a1)	0.40	0.40
CCSM147 (Citrus)	F – GCTATGTTATGATACGTCTG R – AGACTCACGTAACCTACTTC	140 (a1) 150 (a2)	0.64	1.28
TTA15 (Citrus)	F-GAAAGGGTTACTTGACCAGGC R-CTTCCCAGCTGCACAAGC	200 (a1)	0.99	0.99
TTA27 (Citrus)	F-GGATGAAAAATGCTCAAAATG R-TAGTACCCACAGGGAAGAGAGC	200 (a1)	0.40	0.40
MEST 431 (Citrus)	F-GTGATTGCTGGTGTCGTT R-AACAGTTGATGAAGAGGAAG	200 (a1)	0.14	0.14
TS24 (Chick pea)	F-GTAGAAAGAAAAGTACATGGTTGAG R-GCCTAACCCAATAATACCTTCTTTT	220 (a1)	0.85	0.85
TS29 (Chick pea)	F-AACATTCATGAACCTACCTCAACTTA R-CCATATGAGTACACTACCTCTCGG	200 (a1)	0.52	0.52
TS47 (Chick pea)	F-GTTAATATTTTCCGCTTCGT R-TCAAATTGTGTTAAAAATCAAAGTGTT	200 (a1) 300 (a2)	0.89	1.78

#PIC= Polymorphic Information Content, MI= Marker Index

a1 and a2 in parenthesis indicate the allele size generated by the respective SSR markers

genetic similarity between lines ranged 0 to 0.9. The present study revealed that all the 13 genotypes were grouped into 2 major clusters, I and II (Fig. 1). Cluster I comprised of the parental line, while cluster II included both the mutants and the clonal selections. Further, cluster II was divided into two subclusters, IIA and IIB. Subcluster IIA was again categorized into IIA (i), comprising of mutants 5 and 15 Gy, and IIA (ii) comprised of mutants 0.05 and 0.5% EMS, and clonal selection 2/4 EEA. Likewise, subcluster IIB further divided into IIB (i) and IIB (ii). Two distinct subgroups were formed under IIB (i); IIB (i) a consisted of mutants 10 and 20 Gy; while, in contrast, IIB (i) b consisted of only clonal selections, *i.e.*, 1/4, 4/8 and 2/20 EEA. The remaining genotypes, such as, mutants 0.1 and 0.2% EMS fell into subgroup IIB (ii). A critical examination of this dendrogram clearly indicated that the grouping was primarily based on mutagenic treatment with few exceptions. Thus, the parental line not treated with mutagen remained in a separate cluster. The clonal selections grouped distinctly as a separate tree in sub-cluster IIB (i), while the mutation treatments grouped separately. Among the mutation treatments too, physical mutants grouped separately from that of chemical treatments.

The SSR-based PCoA clustered the accessions in close agreement with the UPGMA-based tree. The first three coordinate axes accounted for 67.86% of the variation observed (Fig. 2). The first axis explained 32.08% of genetic variation, while the second axis 21.43% and the third axis 14.35%. The first half of the coordinates had mostly clones grouping together. The second half consisted of genotypes developed through

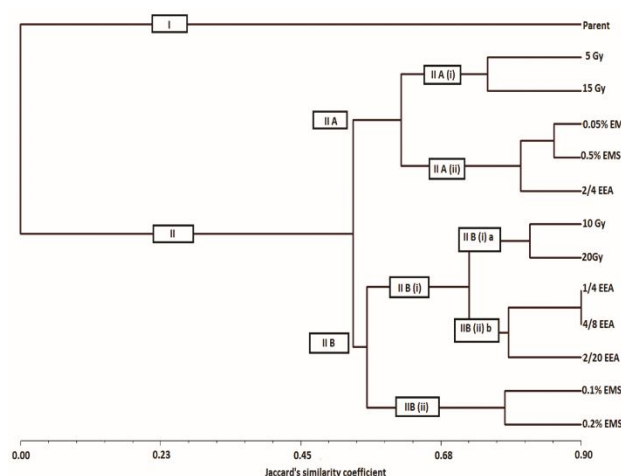


Fig. 1 — UPGMA dendrogram showing genetic relationship among Kinnow genotypes (clones and mutants) based on microsatellite markers derived from citrus and chickpea.

chemical mutagens. Further, AMOVA was used to estimate the partitioning of genetic variance among and within populations (Table 2). Accordingly, the percentage of genetic variation within population was 80%, while it was 20% among the population (Fig. 3).

In the present study, “null” alleles were also observed, which could be due to mutations in the primer binding site leading to non-amplification. Mutations could have occurred in the parental stock at a specific STMS locus, leading to two different alleles on homologous chromosomes. Another possible cause of variation in the germplasm lines is the presence of active mobile elements^{20,21}. The occurrence of heterozygosity in pure lines revealed through STMS analysis could possibly draw the attention of the Kinnow breeders that selection is possible within a Kinnow line using soma clonal variation or through mutation treatment. Thus, molecular characterization can give very useful information to Kinnow breeders. SSRs are considered to be good candidates for classifying genotypes in different groups and thereby assessing genetic distances as well as genetic expected gains.

In the present study, no clear cut pattern, especially for different clustering (genetic dissimilarities) and source population diversity could be found. Mutagenic information might not be a clear cut indicator of diversity and large variation can exist between the genotypes derived from the same mutagenic source.

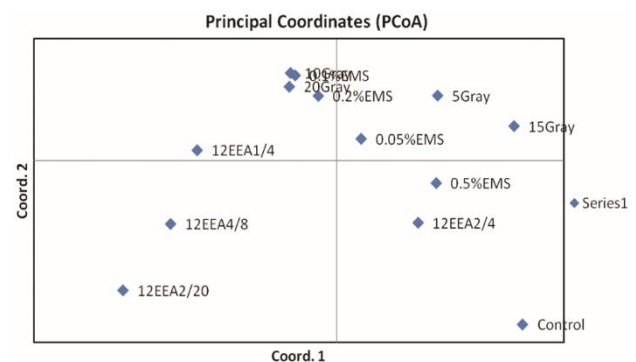


Fig. 2 — PCA of Kinnow genotypes (clones and mutants) based on SSR markers data.

Table 2 — AMOVA summary of SSR markers for Kinnow genotypes (clones and mutants)

Source	Df	SS	MS	Estimated variation	% Variation
Among Pops	2	26.662	13.331	1.614	20%
Within Pops	10	63.800	6.380	6.380	80%
Total	12	90.462		7.994	100%

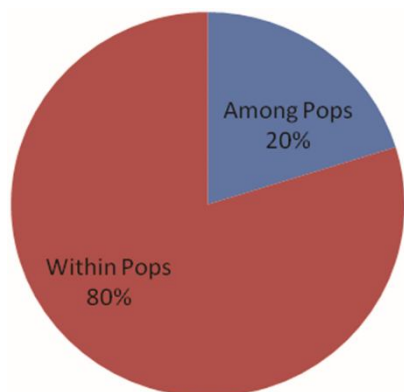


Fig. 3 — AMOVA of Kinnow genotypes (clones and mutants) based on SSR markers data.

This could be probably because mutation is a random event and the diversity generated from a mutagen is not uniform among the treatments. Mutagenic information might not be a clear cut indicator of diversity and large variation between the genotypes could be derived from the same source. This could be possible due to no trait observed, less number of STMS markers used and greater molecular changes in the genotypes under study. In addition, DNA marker may be affected by selection, drift and mutation, factors that are ignored when estimating association among genotype based on pedigree or origin.

The present analysis gives an insight of the interrelationship among the genotypes and highlights the urgency for effective supplementation of morphological data with the database generated by STMS markers to efficiently unearth the genetic interrelationship among the genotypes. It may represent alleles identical in state, which may not be identical by descent²². Finally, it can also be an outcome from the clustering process whenever clusters are non-overlapping. Because of the latter, a genotype related to other genotypes from separate clusters will only be grouped with the closest one²¹. Author has also worked on leaf morphology and found that mutations generally produce leaf variations and thus the pronounced effects are generally seen in leaf structure and characters. The clustering of Kinnow mutants and clones for some extent, indicates their affinity within a cluster to their leaf sclerophylly. The present study clearly demonstrates the utility of microsatellite markers for molecular characterization of 13 Kinnow genotypes. Chickpea markers, though less in number, have successfully amplified polymorphic products in Kinnow genotypes and found to be quite informative. Transferability of microsatellite markers among

Myrtaceae species has been studied to obtain population genetics data to help the conservation of forest tree species²³. However, in citrus this is the first report on cross-genera transferability of microsatellite markers developed in chickpea to 13 Kinnow genotypes comprising of parent, clonal selections and mutants. From the cluster analysis, it is clearly evident that the genotypes under cluster II consisting of mutants and clonal selections appear to be most divergent and farthest apart from the cluster I, i.e., parental genotype. Further, clonal selections, physical and chemical mutants under cluster II have formed their separate sub-clusters showing close genetic relatedness among them. Hence, the present study, clearly brings out the effectiveness of both clonal selection and mutagenic treatment in generating variability over the source of parental material.

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