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Molecular characterization of Kinnow mutants

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ABSTRACT

The present experiment was conducted during 2016-18 at Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi. A total of 40 Kinnow mutants and 1 wild type were selected for molecular characterization and diversity analyses. Thirty-four SSR markers were screened for polymorphism, of which only 2 were found informative. Twelve alleles were detected among 2 SSRs with an average of 6 alleles per locus and the highest number of alleles (7) was recorded in SSR locus AMB2. The average diversity indices of SSR, viz. allele frequency, gene diversity, observed heterozygosity and PIC were 0.415, 0.672, 0.415 and 0.611 respectively. The N-J tree was constructed based on the 2 SSRs data which clustered the mutants into 2 major groups. Subsequently, clusters were simplified into 6 clades which distinguished gamma-irradiated and EMS derived mutants and results were reconfirmed through principal coordinate analysis (PCoA). First three axes of PCoA contributed 80.88% of the cumulative variation among the Kinnow mutants. The analysis of molecular variance (AMOVA) explained 42% variation among the populations, 48 and 10% variation within and among the individuals respectively. The present investigation genetically characterized the Kinnow mutants and deciphered the genetic diversity among them. Thus, the variability generated through induced mutagenesis could be used as valuable genetic material for Kinnow improvement.

Keywords: AMOVA, EMS, Gamma irradiation, Induced mutants, Kinnow mandarin, SSRs

Kinnow mandarin has changed the scenario of the citrus industry in India. The fruit has gained popularity owing to its precocious and prolific bearing habit, attractive orange-coloured fruits with improved fruit quality and better economic return (Kumar *et al*. 2018). Regardless of numerous positive traits, some inbuilt characteristics like vigorous plant growth, its susceptibility to various biotic and abiotic stresses and the presence of 30-35 seeds per fruit that makes the juice unsuitable for processing. Thus, dwarf tree size, seedlessness and disease resistance which contribute to increased yield and quality are the prime objectives of Kinnow improvement. To fulfil the growers and consumer's preferences, conventional and non-conventional breeding approaches are commonly used which has its advantages and limitations. Although promising varieties have been developed in *Citrus* through conventional breeding, this approach is restricted by the complication of their genetic system, specific reproductive biology, facultative apomixis, polygenic nature of many important traits, embryo abortion and overall long juvenile phase. Breeding efforts through mutagenesis on the other hand have resulted in the development of several seedless mutants in citrus such as Star Ruby a deep red fleshed seedless grapefruit. Mutagenesis has also been used to improve other characteristics such as spine free mandarin and Mal Secco tolerance in lemon (Gulsen *et al*. 2007).

The knowledge about genetic diversity, relatedness within and between the mutant population of citrus and their wild type are of importance to ensure long term success in the crop improvement programme. In this direction biotechnological tools such as molecular markers have emerged as a powerful tool for accelerating the process of varietal identification. Among molecular markers, Simple sequence repeats (SSR), a type of microsatellite marker, are particularly useful for characterization of germplasm/mutant population because they are highly polymorphic, usually co-dominant and easy to use. SSRs have been used in citrus for a wide range of applications like identification of clones and mutants, determining genetic diversity, construction of linkage maps, fingerprinting etc. (Corazza-Nunes *et al*. 2002, Barkley *et al*. 2006). Thus, in the present investigation, simple sequence repeats (SSRs) markers were used for molecular characterization and genetic diversity analyses among the Kinnow mutants.

MATERIALS AND METHODS

Plant material: The present study was carried out during 2016-18 at Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi. A total of forty induced mutants

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of Kinnow mandarin and one wild type were selected for molecular characterization and their genetic diversity analyses. Kinnow mutants were developed through the treatment of different doses of gamma irradiation (15, 20, 25 and 30 Gray) and ethyl methanesulphonate (EMS) (0.05%, 0.1%, 0.2% and 0.5%) (Table 1). The resultant mutants were raised on *Jatti Khatti* rootstock and maintained with uniform cultural practices.

SSR markers: Thirty-four SSR primer sets were selected from research articles published by researchers (Barkley *et al*. 2006; Ollitrault *et al*. 2010, Altaf *et al*. 2014, Mallick *et al*. 2017). The criteria for selection of these SSRs were their high PIC value among the studied *Citrus* species/ genotypes. The selected primers were synthesized from Integrated DNA Technologies, Inc. Coralville, Iowa (USA).

Extraction of genomic DNA: Young leaves were collected from mutants and wild type, and frozen immediately in liquid nitrogen and stored in deep freezer refrigerator (-80°C) until further use. Total genomic DNA was extracted from the stored leaf tissue following the standard Cetyl Trimethyl Ammonium Bromide (CTAB) method which was described by Doyle and Doyle (1990) with some minor modifications.

Quantification of genomic DNA: The quality and quantity of DNA were determined using mySPEC microvolume spectrophotometers (VWR International Ltd., USA) and 0.8% agarose gel. Accordingly, the final concentration of purified DNA was maintained at 20 ng/ µl in nuclease-free water as working dilution and kept in deep freeze (-20°C) until the further use.

PCR amplification: A PCR reaction mixture of 10 µl was prepared using 5.0 µl of red dye PCR master mix (GeNei™, India), 1.0 µl of each of forward and reverse primer, 2.0 µl of DNA (40 ng) and 1 µl of nuclease-free water. Thermo cycle of the PCR (C1000 Touch™ thermal cycler, Bio-Rad Laboratories, Inc., USA) consist with an initial denaturation at 94°C for 4 min and subsequent 35 cycles, each with denaturation at 94°C for 1 min, primer annealing temperature varied from 48-57°C (Specified with gradient temperature in PCR for each primer) for 1 min and primer extension at 72°C for 1 min. The final extension step was performed at 72°C for 7 minutes. PCR-amplified DNA products were separated on a 4.5% agarose gel containing 1X TBE and 0.5 µg/ml aqueous solution of ethidium bromide. The agarose gel was run at a constant voltage of 100V for 4–5 h in 0.5X TBE buffer. The gels were visualized under UV light and photographed using photo gel documentation system (C200 Azure™ Biosystems, USA). A 100 bp molecular weight marker (DNAmark™, G-Biosciences, USA) was used for estimation of allele sizes.

Data scoring and statistical analyses: The amplified fragments of SSR loci were scored manually among the Kinnow mutants including the wild type. Based on the scored data, the genetic diversity indices, viz. the number of alleles, major allelic frequency, gene diversity or expected heterozygosity (Nei 1972), observed heterozygosity and polymorphism information content of each SSR locus were calculated using the tool, PowerMarker v3.25 (Liu and Muse

Table 1 Description of Kinnow mutants developed through gamma irradiation and EMS

Gamma irradiation dose	Mutant population	Mutant code	EMS concentration	Mutant population	Code of mutant
Non treated	Wild type	WT	Non treated	Wild type	WT
15 Gray	15-1	G_1	0.05 %	0.05-1	E_1
15 Gray	15-2	G_2	0.05 %	0.05-2	E_2
15 Gray	15-3	G_3	0.05 %	0.05-3	E_3
15 Gray	15-4	G_4	0.05 %	0.05-4	E_4
15 Gray	15-5	G_5	0.05 %	0.05-5	E_5
20 Gray	20-1	G_6	0.1 %	0.1-1	E_6
20 Gray	20-2	G_7	0.1 %	0.1-2	E_7
20 Gray	20-3	G_8	0.1 %	0.1-3	E_8
20 Gray	20-4	G_9	0.1 %	0.1-4	E_9
20 Gray	20-5	G_{10}	0.1 %	0.1-5	E_{10}
25 Gray	25-1	G_{11}	0.2 %	0.2-1	E_{11}
25 Gray	25-2	G_{12}	0.2 %	0.2-2	E_{12}
25 Gray	25-3	G_{13}	0.2 %	0.2-3	E_{13}
25 Gray	25-4	G_{14}	0.2 %	0.2-4	E_{14}
25 Gray	25-5	G_{15}	0.2 %	0.2-5	E_{15}
30 Gray	30-1	G_{16}	0.5 %	0.5-1	E_{16}
30 Gray	30-2	G_{17}	0.5 %	0.5-2	E_{17}
30 Gray	30-3	G_{18}	0.5 %	0.5-3	E_{18}
30 Gray	30-4	G_{19}	0.5 %	0.5-4	E_{19}
30 Gray	30-5	G_{20}	0.5 %	0.5-5	E_{20}

Table 2 The genetic diversity indices of polymorphic SSR loci used for the genetic characterization of Kinnow mutants

Marker	Major allele frequency	Genotype no	Sample size	Allele no	Gene diversity	Heterozygosity	PIC
AMB2	0.341	8.000	41.000	7.000	0.752	0.415	0.713
CCSM40	0.488	6.000	41.000	5.000	0.593	0.415	0.509
Mean	0.415	7.000	41.000	6.000	0.672	0.415	0.611

2005). The Neighbour-joining tree was also constructed using the phylogeny option in PowerMarker v3.25. Further, analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA) was analysed using GenAlEx v6.5 (Peakall and Smouse 2006).

RESULTS AND DISCUSSION

Molecular characterization using SSR markers: A total of 34 SSRs were screened of which only 2 were found polymorphic among the studied Kinnow mutants. Out of the 34 SSRs, 5 did not produce amplicon while 27 SSRs gave monomorphic alleles. The diversity indices, viz. the number of alleles, major allelic frequency, expected heterozygosity, observed heterozygosity and polymorphic information content of two polymorphic loci were calculated (Table 2). The allelic size ranged from 260 to 300 bp among the 2 SSR loci. A total of 12 alleles were amplified with an average of 6 alleles at per locus and the highest number of alleles (7) was amplified by SSR, AMB2. The major allelic frequency of the SSR loci ranged from 0.341 (AMB2) to 0.488 (CCSM40) with an average of 0.415. The gene diversity varied from 0.593 to 0.752 among two SSR loci with an average of 0.672. The observed heterozygosity for both the SSR locus was 0.415. The average PIC value was 0.611 and the highest PIC (0.713) was recorded for locus AMB2. A total of 12 alleles were amplified of which 4 alleles had frequency less than 0.05 and that was amplified with only single Kinnow mutant. Thus they were considered as rare and unique alleles, respectively.

In the past, SSR markers have been used for the molecular characterization of the *Citrus* species/genotypes. For instances, Barkley *et al*. (2006) characterized, 370 accessions of citrus accession using 24 SSR markers and found an average PIC value of 0.625 per locus. Similarly, Jannati *et al*. (2009) characterized 27 *Citrus* genotypes including the bud mutants using 15 SSRs. Recently, Singh *et al*. (2016) assessed the genetic diversity and fingerprint, 19 mandarin genotypes using 60 SSRs. They recorded an average PIC value of 0.40 and number of alleles 2.46 per locus. The present study also illustrated the substantial number of polymorphic alleles and PIC value of selected SSRs among the Kinnow mutants. Thus, these SSR markers could be informative for the characterization of induced mutants in citrus. Further, the occurrence of unique alleles or rare SSR alleles is essential to maintain the genetic diversity and present study depicted 4 of each rare and unique allele by 2 SSRs among the Kinnow mutants. The resources of SSR alleles may be an indication of addition or deletion of the small number of repeats (Goldstein and Pollock 1997) and most rational explanation for high mutation rate is polymerase slippage (Levinson and Gutman 1987). Our findings also indicate that polymorphism resulting from SSR markers, which might be associated with genetic variability in the mutants, thus showed that microsatellites can distinguish mutation-derived variability. The use of multi-locus, PCR-based SSR markers allowed efficient differentiation of tightly linked accessions. These results are in accordance with the finding of Hvarleva *et*

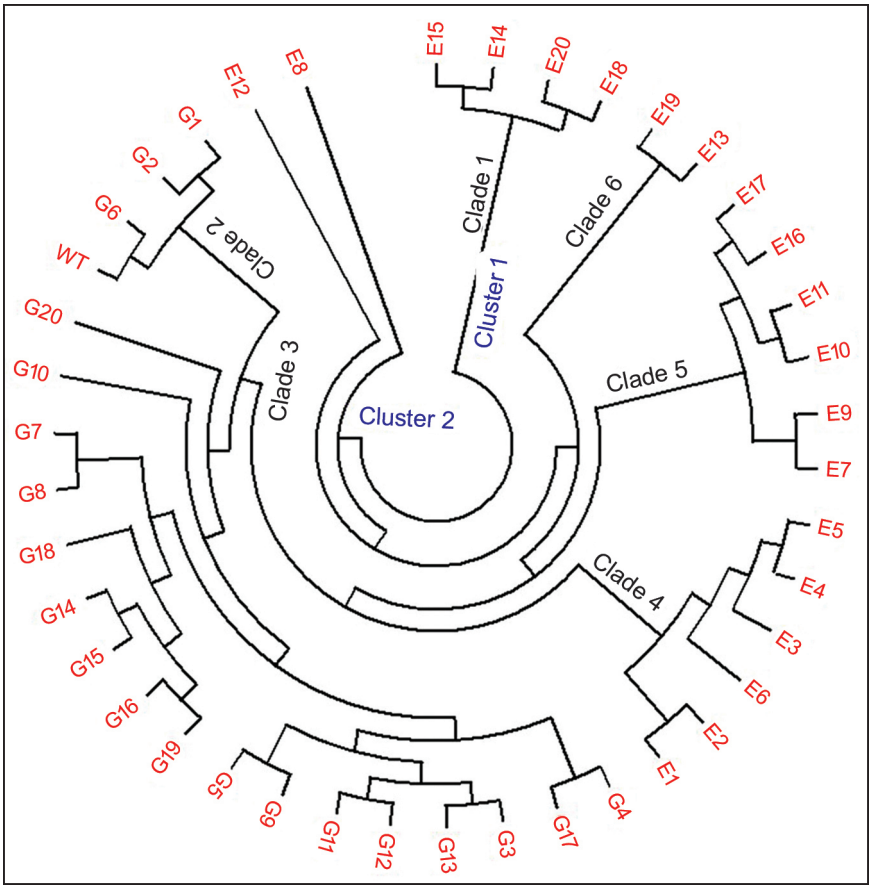

Fig 1 The N-J tree of wild type and Kinnow mutants based on SSR loci.

al. (2008) and Polat *et al*. (2012).

Cluster analysis: The phylogenetic relationship among the Kinnow mutants including the wild type, was established using the Neighbour-joining (N-J) tree based on the SSRs data. The N-J tree genetically clustered Kinnow mutants and divided into 2 major groups which were further simplified into 6 clades (Fig 1). The Clade 1 constituted by Kinnow mutants, *viz*., E_{15}, E_{14}, E_{20} and E_{18}; Clade 2 by G_1, G_2, G_6 and wild type; Clade 3 by G_{20}, G_{10}, G_7, G_8, G_{18}, G_{14}, G_{15}, G_{16}, G_{19}, G_5, G_9, G_{11}, G_{12}, G_{13}, G_3, G_{17} and G_4; Clade 4 had E_1, E_2, E_6, E_3, E_4 and E_5; Clade 5 retained mutants like E_7, E_9, E_{10}, E_{11}, E_{16} and E_{17}; Clade 6 had E_{13} and E_{19}. Earlier, the cluster analysis was used for the genetic classification and phylogenetic analysis of the *Citrus* accessions. For instances, Barkley *et al*. (2006) studied the phylogenetic relationships among *Citrus* accessions and putative non-hybrid *Citrus* using Neighbour-joining trees based on SSR markers. Similarly, 45 *Citrus* genotypes were genetically grouped using the Neighbour-joining tree constructed based on the SSR markers (Ollitrault *et al*. 2010). Further, Polat *et al*. (2012) illustrated the genetic relationship and magnitude of genetic diversity among the sour orange accessions and their relatives using the cluster analysis. Thus, the present investigation enables to genetically classify the Kinnow mutants derived through the gamma irradiations and EMS using the N-J tree based on SSRs. The genetical grouping of the Kinnow mutants was in accordance with their mutagen used.

AMOVA and principal coordinate analysis: The analysis of molecular variance (AMOVA) was done using the two population groups. Considering the results of N-J tree, the Kinnow mutants were grouped into two groups that the first group constituted by 20 Kinnow mutants derived through gamma irradiation and one wild type while the second group by 20 EMS induced mutants. The AMOVA revealed 42% variation between the populations; whereas 10 and 48% variation were noted among and within the individuals of Kinnow mutants respectively which deciphered substantial molecular variance among them. The first three axes of the principal coordinate analysis explained 80.88% cumulative variance, thus indicating high genetic diversity among the studied Kinnow mutants. In PCoA, Kinnow mutants labelled with two colours represented in two different groups of Kinnow mutants, across the coordinates (Fig 2) which also reconfirmed the results of N-J tree. Earlier, Mallick *et al*. (2017) elucidated the pattern of grouping of gamma-irradiated and EMS induced mutants using the PCoA and also molecular variance using the AMOVA. Our findings suggested that the Kinnow mutants used in the present study were categorized according to their mutagenic treatment, i.e. gamma irradiation and chemical mutagen, EMS.

In this study, microsatellites (SSR) markers revealed considerable genetic diversity among the Kinnow mutants developed through gamma irradiation and EMS. Out of 34 SSRs, only 2 were found polymorphic and these loci are valuable for further identification and characterization of induced mutants in mutation breeding programmes. The study also indicated that by using induced mutation, the genetic diversity of a superior crop/variety could be enhanced for selection and improvement. Genetic diversity present in the mutants will enable detection of the efficient mutant for prospective exploitation in Kinnow improvement.

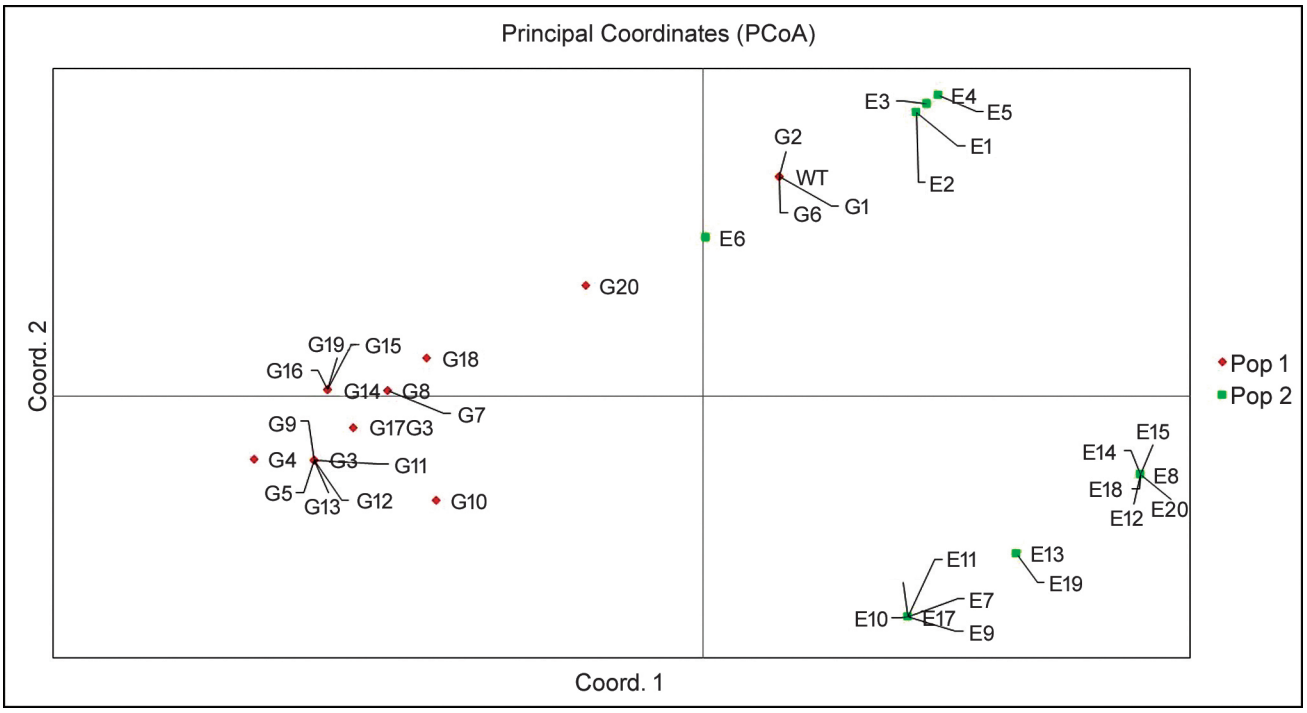

Fig 2 The principal coordinate analysis of Kinnow mutants based on SSR loci

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