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Genetic diversity and population structure of sweet orange (*Citrus sinensis*) germplasm revealed by SSR and InDel markers

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ABSTRACT

An experiment was conducted during 2019–21 at ICAR-Central Citrus Research Institute, Nagpur, Maharashtra, in which two molecular markers, SSR and InDels were used to understand the genetic diversity and population structure of 72 sweet orange [*Citrus sinensis* (L.) Osbeck] genotypes. The studied genotypes showed a genetic variability with an average genetic polymorphism of 68.89 and 56.52% with InDels and SSR, respectively. Allele numbers ranged from 1–2 with 1.86 and 1.25 average numbers of alleles per marker for InDel and SSR, respectively. Two dendrograms were constructed independently based on the InDels and SSR markers. In both cases, they formed 3 major clusters showing various degrees of variations concerning members of the clusters. Based on k-means clustering, the studied genotypes were divided into 2 distinct sub-populations. Soh Khylla, Soh Niairang, and Tasi, the genoptypes from north-eastern region exhibited informative and distinctive patterns in both the marker types.

Keywords: Citrus, Diversity, Genetic variation, InDel markers, SSR markers, Sweet orange

Sweet orange [*Citrus sinensis* (L.) Osbeck] dominates the global citrus consumption, constituting around 38% of citrus area and 48% of citrus production (FAO 2022). Brazil leads in sweet orange production, trailed by India and China (FAO 2022). In India, sweet orange ranks third after mandarins and limes, covering 0.190 million hectares and yielding 3.483 million metric tonnes (NHB 2018–19). Sweet orange, draft genome sequenced (Xu *et al.* 2013), has been identified as a natural hybrid of pummelo (*C. maxima*) and mandarin (*C. reticulata*) (Wu *et al.* 2018). Exploring the intricacies of citrus taxonomy involved phylogenetic studies based on morphological and physiological traits (Gupta *et al.* 2018), DNA markers (Lamine and Mliki 2015, Polat 2015), chloroplast genomes (Carbonell-Caballero *et al.* 2015), and whole genomes (Wu *et al.* 2014, Wu *et al.* 2018) of various citrus species, including sweet orange. While extensive work on sweet orange germplasm has been conducted globally (Xu *et al.* 2013, Caruso *et al.* 2018, Abouzari *et al.* 2020), India's contributions remain limited to RAPD markers (Malik *et al.* 2012, Sankar *et al.* 2014), revealing challenges such as naming of variants leading to synonyms and duplicates.

Advancements in low-cost next-generation sequencing (NGS) technologies, such as SSR, SNP, and InDel markers, have significantly enhanced marker identification (Taheri *et al.* 2018). Within citrus species, SSR markers have been developed from diverse sources, including genomic sequences (Xu *et al.* 2013), expressed sequence tags (ESTs) (Chen *et al.* 2008), and BAC end sequences (Terol *et al.* 2008). Various InDel markers have been identified in different citrus species (Ollitrault *et al.* 2012, Fang *et al.* 2018).

In this study, a comprehensive analysis was conducted on a dataset comprising 72 sweet orange accessions, employing SSR and InDel markers for their molecular characterization. The primary aim of this investigation was to unravel the phylogenetic relationships and assess the genetic variability within the collected sweet orange germplasm.

MATERIALS AND METHODS

Plant material and collection: The experiment was conducted during 2019–21 at ICAR-Central Citrus Research Institute, Nagpur, Maharashtra. For molecular investigations, a collection of 72 sweet orange cultivars (Table 1) was sourced from the National Active Germplasm site at ICAR-Central Citrus Research Institute, Nagpur, Maharashtra.

DNA extraction: Total genomic DNA was extracted from all the 72 cultivars of sweet orange by new just matured leaves using cetyl tri-methyl ammonium bromide (CTAB) method (Doyle 1991) with some modifications. DNA was extracted with a standard CTAB protocol and the samples were diluted with with 1XTE to 50 ng/µl for PCR reactions.

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Table 1 Details of sweet orange accessions

Name of the germplasm	IC/EC No.	Source of origin
M1	IC-311476	Jadgaon, Aurangabad, Maharashtra
M2	IC-311478	Dawarwadi, Aurangabad, Maharashtra
M3	IC-311479	Paithan, Aurangabad, Maharashtra
M4	IC-311480	Paithan, Aurangabad, Maharashtra
M5	IC-311481	Paithan, Aurangabad, Maharashtra
M6	IC-311482	Paithan, Aurangabad, Maharashtra
M7	IC-311483	Paithan, Aurangabad, Maharashtra
M8	IC-311484	Dawarwadi, Jalna, Maharashtra
M9	IC-311485	Talegaon, Jalna, Maharashtra
M10	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M11	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M12	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M13	IC-311489	Guha, Ahamadnagar, Maharashtra
M14	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M16	IC-311490	Adhawadi, Ahamadnagar, Maharashtra
M17	IC-311491	Kolgaon, Ahamadnagar, Maharashtra
M18	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M24	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M25	IC-311493	Kolgaon, Ahamadnagar, Maharashtra
M26	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M27	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M28	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M30	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M32	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M33	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M34	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M36	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M37	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M38	IC-311494	Kolgaon, Ahamadnagar, Maharashtra
M39	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M41	IC-311495	Kolgaon, Ahamadnagar, Maharashtra
M42	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M43	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M44	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M45	IC-311498	Talegaon, Pune, Maharashtra
M46	IC-311501	Shokrapur, Pune, Maharashtra
M51	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M52	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M53	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M55	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M57	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M60	IC-311502	Usthaldumali, Ahamadnagar, Maharashtra
M63	IC-311503	Usthaldumali, Ahamadnagar, Maharashtra
M64	IC-311504	Kaygaon, Aurangabad, Maharashtra
M65	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M66	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M67	IC-311505	Kaygaon, Aurangabad, Maharashtra
M68	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M69	IC-322089	Baruasagar, Jhansi, Uttar Pradesh
M70	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M74	IC-322097	Baruasagar, Jhansi, Uttar Pradesh
M75	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra

Contd.

Table 1 *Concluded*

Name of the germplasm	IC/EC No.	Source of origin
M77	IC-322247	Mahatargaon, Hingoli, Maharashtra
M80	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M81	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M152	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M153	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M156	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M157	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
M159	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
Phule Mosambi	Obtained from State Agricultutral University	Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra
Kodur sathgudi	Obtained from State Agricultutral University	Citrus Research Station, Tirupati (Dr. YSR Horticultural University), Andhra Pradesh
Soh Bittara	IC- 344929	Barapani, Ribhoi, Meghalaya
Mosambi	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
Katol Gold	Obtained from State Agricultutral University	Dr. Panjabrao Deshmukh Krishi Vidyapeeth, PDKV, Akola, Maharashtra
Sathgudi _132	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
Tasi	IC- 344927 / IC-346977	Arunachal Pradesh
Soh khylla	IC-344935	Umroi, Ribhohi, Meghalaya
Soh nairiange	IC- 346992 / IC-344928	Basar, West Siang, Arunachal Pradesh/Barapani, Ribhoi, Meghalaya
Nucellar	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
TM-33	Obtained through clonal selection	ICAR-CCRI, Nagpur, Maharashtra
Sathgudi	IC-322250	Limbgaon, Nanded, Maharashtra

PCR amplification and visualization: 90 SSR primers were meticulously chosen based on their specific chromosome locations (Supplementary Fig. 1). Subsequently, the 46 SSR primers, along with 45 InDel primers selected for their chromosomal locations, underwent screening specifically in sweet orange accessions. Amplification reaction were performed in the volume of 20 µl containing 2 µl 10X assay buffer, 1.6 µl of dNTPs, 1 µl of Taq DNA polymerase (1 u/µl), 1 µl of DNA primer and 2 µl of template DNA (50 ng/µl). The PCR amplification conditions were as follows: initial denaturation at 96ºC for 5 min followed by 45 cycles of denaturation for 30 sec @96ºC, annealing @58ºC (for SSR) and 55ºC (for InDel) for 40 sec and extension @72ºC for 1 min followed by final extension at 72ºC for 10 min. The resulting amplified products underwent electrophoresis (70 V for 3 h) in a 4% agarose gel containing ethidium bromide. Gel images were visualized using a UV gel documentation system.

Data analysis: The alleles of each primer locus were scored with 100 bp DNA ladder (Genexy). The statistics such as number of alleles per locus, polymorphism and genetic diversity of each primer were calculated. The polymorphic information content (PIC) and heterozygosity were also calculated for each primer by using the method described by Chesnokov and Artemyeva (2015). The genetic dissimilarity matrix was calculated using simple matching dissimilarity index among pairs of accessions. An Unweighted neighbor-joining tree was constructed from calculated dissimilarity matrix to depict the genetic relationship among the individuals using the DARwin software version 6 (Perrier and Jacquemoud-Collet 2006) and the firmness and tenacity of branches was evaluated using 1000 bootstraps.

The STRUCTURE software version 2.3.4 (Pritchard *et al.* 2000) was used for the determination of the most probable number of clusters for population structure (K value). Using the admixture model, 8 simulations were performed for each inferred K value, with a running length composed of 300,000 burning periods and 50,000 Markov chain Monte Carlo (MCMC) iterations to allocate accessions to different populations. The output from this analysis was then used as input in the Structure HARVESTER online programme to determine the optimal K value (Earl and VonHoldt 2012).

RESULTS AND DISCUSSION

Genetic diversity analysis: A total of 72 accessions in sweet orange germplasm were subjected to scrutiny using a diverse set of molecular markers, including 45 Insertion-Deletion (InDel) markers and 46 Simple Sequence Repeat (SSR) markers. The examination of 91 molecular markers revealed intriguing insights into the genetic landscape of sweet orange. InDels, originating from transposable element insertions, slippage in simple sequence replication, or unequal crossover events, offer advantages such as simple amplicon lengths, high transferability, absence of stutter, low mutation rates, and widespread distribution across the genome (Ollitrault *et al.* 2012, Fang *et al.* 2018).

Out of the exhaustive marker set, 34 primers, consisting of 14 InDel (31.11%) and 20 SSR (43.48%) markers, resulted in the amplification of 51 polymorphic amplicons. In contrast, 57 markers (31 InDel - 68.89%, 26 SSR - 56.52%) led to the amplification of monomorphic products, indicating a varied genetic makeup within the sweet orange germplasm. The observed allele number ranged from 1–2, with InDel markers displaying an average of 1.86 alleles

Table 2 Polymorphic analysis of InDel primers used in sweet orange

InDel primer	Total no. of amplicon	Poly. alleles	Polymorphism (%)	PIC	G.D.	Heterozygosity
IND-chr3-9113	2	1	50	0.08	0.08	0.08
IND-chr3-78081	1	1	100	0.41	0.42	0.91
IND-chr3-100795	2	2	100	0.09	0.09	0.55
IND-chr4-1894	2	2	100	0.33	0.33	0.37
IND-chr4-24656	2	1	50	0.05	0.05	0.50
IND-chr4-41717	2	2	100	0.04	0.04	0.50
IND-chr4-67425	2	2	100	0.39	0.39	0.46
IND-chr5-7677	2	2	100	0.42	0.42	0.51
IND-chr5-24964	2	2	100	0.12	0.12	0.58
IND-chr6-69785	1	1	100	0.44	0.45	0.56
IND-chr7-2104	2	2	100	0.20	0.20	0.21
IND-chr9-3837	2	2	100	0.48	0.49	0.64
IND-chr1-10341	2	2	100	0.38	0.38	0.45
IND-chr1-43527	2	2	100	0.20	0.21	0.65
Mean	1.86	1.79	96.43	0.26	0.26	0.50

PIC, Polymorphic information content; G.D., Genetic diversity.

per marker, while SSR markers exhibited an average of 1.25 alleles/marker.

An interesting facet of the study was the high polymorphism displayed by the selected InDel and SSR primers, with the exception of IND-chr4-24656, which exhibited 50% polymorphism. Polymorphic Information Content (PIC) values (Botstein *et al.* 1980), reflective of allelic frequency in the population, ranged from 0.04–0.48 for InDel markers (Table 2) and 0.08–0.50 for SSR markers (Table 3). This observation led to the inference of a relatively low genetic diversity in the sweet orange germplasm, substantiating the need for further exploration and conservation efforts. Notably, 7 InDel and 12 SSR markers surpassed the average PIC values within their respective marker categories.

Heterozygosity, a crucial metric reflecting genetic variation, ranged from 0.08–0.91 for InDel markers and 0.08–1.00 for SSR markers. Genetic diversity values exhibited a spectrum from 0.04–0.49 for InDel markers and 0.08–0.51 for SSR markers. Moving beyond the molecular analysis, cluster analyses based on InDel and SSR markers unveiled distinct patterns. InDel markers resulted in a division of the 72 accessions into 3 main clusters, with the largest cluster (Cluster I) encompassing 43 accessions, followed by Cluster II with 25 accessions, and the smallest, Cluster III, containing only 4 accessions (Fig. 1). Further, sub-divisions within Cluster I highlighted the presence of two major clusters (IA and IB) and unveiled unique characteristics of northeastern collections.

On the other hand, SSR data-driven cluster analysis portrayed a different grouping, with 3 main clusters (Fig. 2). The Cluster I contains 36 accessions. The Cluster II contains 30 accessions and the Cluster III contains 6 accessions. The nuances observed in these clusters provide valuable insights into the genetic relationships among sweet orange accessions, setting the stage for future investigations into their origin and traits.

Population structure analysis: Most of the sweet oranges are diploids with a comparatively small genome size of about 367 Mb (Arumuganathan and Earle 1991). Sweet oranges usually show low level of genetic diversity (Caruso *et al.* 2018). After coming through by introduction, most Indian sweet orange accessions originated *via* mutations. Besides, there are some Indian origin collections which show clear separation from the others. The level of polymorphism is very less may be due to the narrow genetic basis and somatic mutations contributing for most of the variation. The genetic diversity of *C. sinensis* is reducing due to selection and introduction of genotypes suitable for intensive horticulture (Agora 2000). The sweet orange genetic resources in India apparently have been subjected to human selection for centuries is creating a genetic bottleneck.

Population structure analysis, a key component of the study, was conducted using calculated membership fractions for different k values ranging from 1 to 6. Intriguingly, the STRUCTURE analysis identified the optimal value of 2 (K = 2), indicating the presence of two sub-populations within the entire accession set (Fig. 3). This observation was further supported by the highest likelihood value at K = 2 in the *adhoc* measure (ΔK) analysis (Supplementary Fig. 2). Sweet orange is a result of the natural cross between pummelo and mandarin (Wu *et al.* 2018). The population structure showing two sub-populations might belong to mandarin and pummelo.

The genotypic data, crucial for understanding genetic diversity and population genetics, was harnessed for the

Table 3 Polymorphic analysis of SSR primers used in sweet orange

SSR primer	Total no. of amplicon	Poly. alleles	Polymorphism (%)	PIC	G.D.	Heterozygosity
Csin-0551	1	1	100.0	0.42	0.43	0.52
Ma3-167	1	1	100.0	0.10	0.11	0.11
Ma2-1766	1	1	100.0	0.08	0.08	0.08
Csin-0100	2	2	100.0	0.35	0.35	0.88
Ma4-51	1	1	100.0	0.48	0.48	0.63
Csin-0167	1	1	100.0	0.42	0.43	0.91
Ma2-94	2	2	100.0	0.41	0.42	0.91
Ma2-1824	1	1	100.0	0.49	0.50	0.68
Csin-0368	1	1	100.0	0.47	0.48	0.61
Ma3-1327	1	1	100.0	0.35	0.35	0.40
Ma3-125	2	2	100.0	0.46	0.47	0.85
Ma2-1162	1	1	100.0	0.35	0.35	0.40
Csin-0149	1	1	100.0	0.48	0.49	0.64
Ma3-122	1	1	100.0	0.31	0.32	0.35
Csin-0514	2	2	100.0	0.43	0.44	0.54
Ma3-5	2	2	100.0	0.13	0.13	1.00
Ma2-1201	1	1	100.0	0.49	0.50	0.68
Ma2-1710	1	1	100.0	0.38	0.38	0.44
Csin-0464	1	1	100.0	0.50	0.51	0.72
Ma2-1856	1	1	100.0	0.49	0.50	0.80
Mean	1.25	1.25	100.00	0.38	0.39	0.61

PIC, Polymorphic information content; G.D., Genetic diversity.

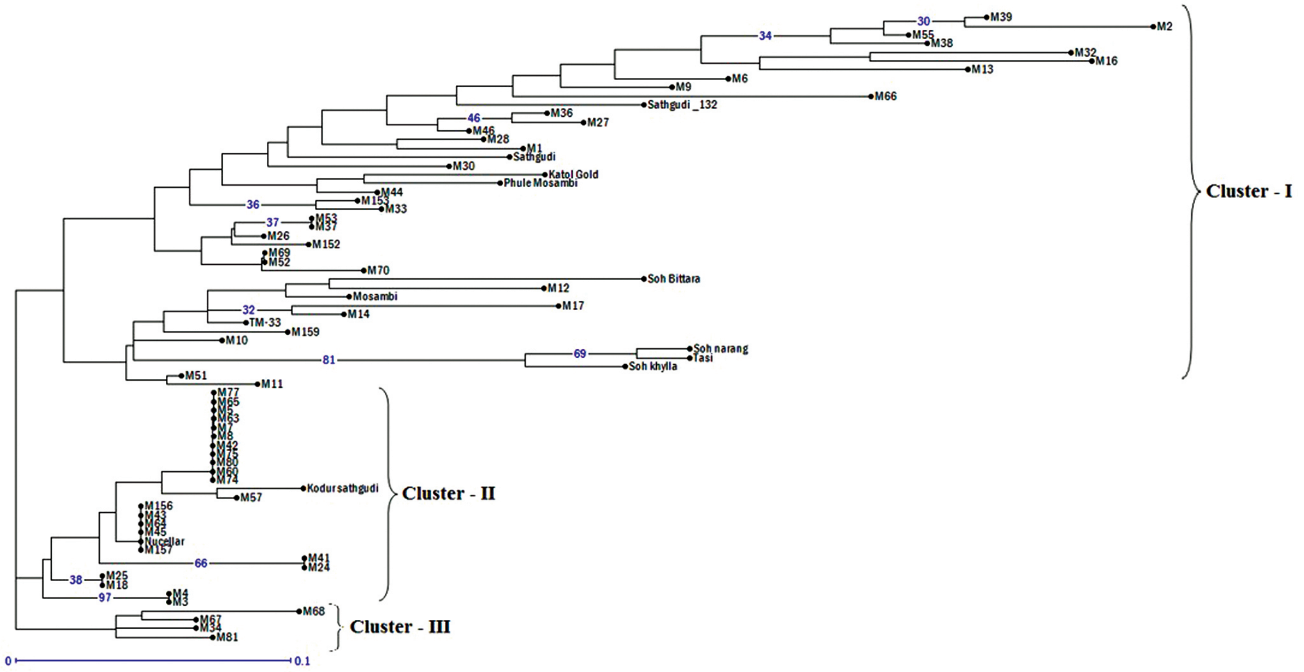

Fig. 1 Dendrogram of 72 sweet orange accessions based on InDels.

overarching goal of shaping future breeding efforts in sweet orange. The study strategically employed InDel and SSR markers, revealing distinct patterns in clusters and providing a foundation for trait mapping and tagging studies. Noteworthy is the observation that most accessions included in the study were either clonal selections or bud mutations, with certain cultivars originating from the northeastern region. The divergence in clusters formed based on InDel

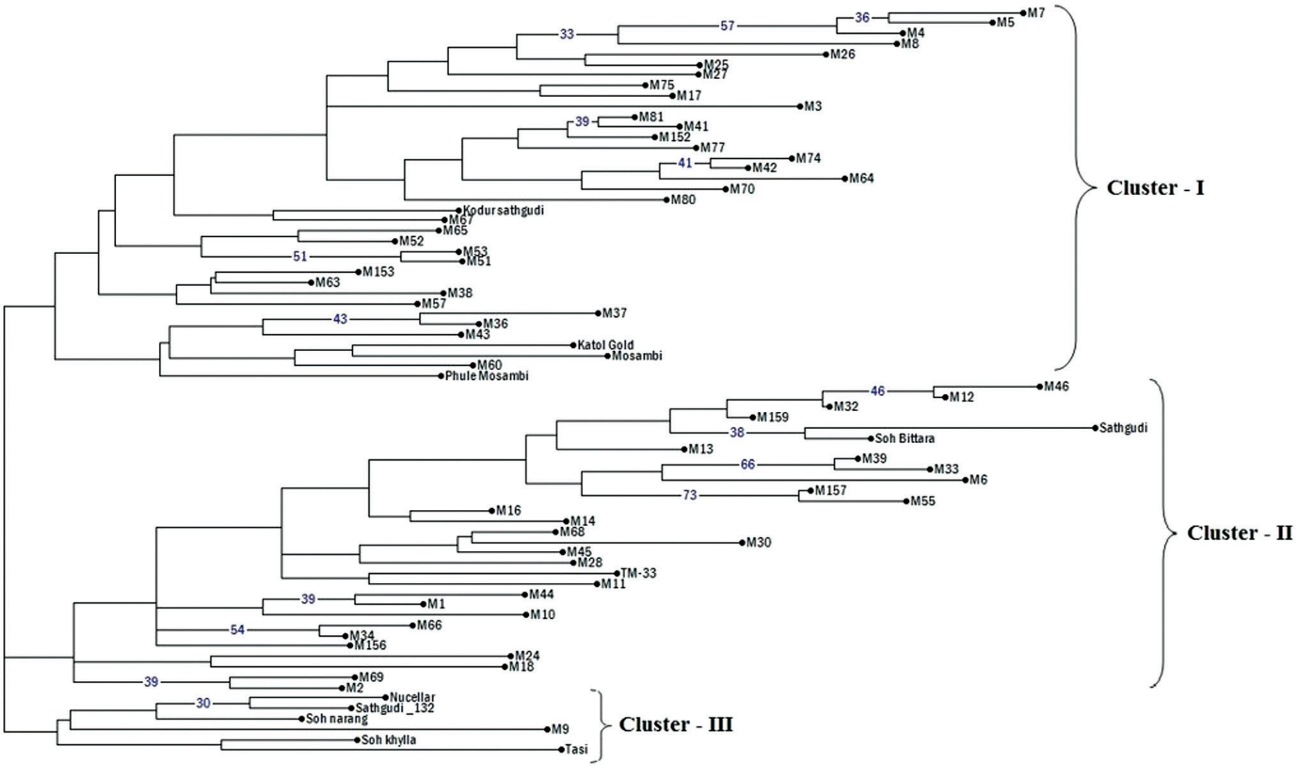

Fig. 2 Dendrogram of 72 sweet orange accessions based on SSR.

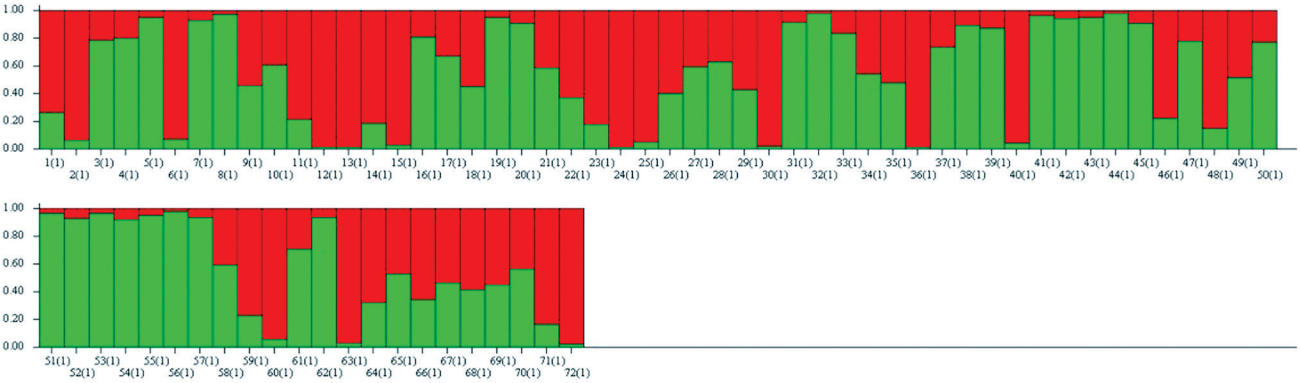

Fig. 3 Population structure of 72 sweet orange accessions based on SSR and Indel markers (K = 2) and graph of estimated membership fraction for K = 2.

and SSR markers, along with the identification of duplicates in InDel clusters, adds a layer of complexity to the genetic relationships among sweet orange accessions.

The presence of clear differences in clusters based on InDel and SSR markers underscores the importance of marker choice in genetic studies. The study draws attention to the potential of markers like Soh Khylla, Soh Niairang, and Tasi, which exhibited informative and distinctive patterns in both marker types. The study notes the prevailing low level of genetic diversity, an aspect that may be attributed to the historical bottleneck caused by human selection and the introduction of genotypes suitable for intensive horticulture. Several online based citrus genetic resources databases were coming up and data from India will help to speed up the breeding processes and to protect the species from extinction (Biswas *et al.* 2020). The study concludes with a forward-looking perspective, highlighting the potential role of molecular characterization in assessing and enhancing genetic diversity in sweet orange. The two sub-populations identified in the population structure analysis are speculated to represent mandarin and pummelo, adding depth to our understanding of the fruit's evolutionary history (Wu *et al.* 2018).

In the present study, the genetic diversity and population structure of sweet orange germplasm resources were analyzed using SSR and InDels. The results showed they have abundant genetic diversity. However, the results vary with respect to SSR and Indels. With area increasing in the sweet orange, it’s time we work towards the coloured sweet oranges and varieties suitable for processing. The outcomes of this study bear significance in discerning the intricate genetic relationships, facilitating the development of a core collection, formulating targeted breeding programmes, and validating the authenticity of newly developed cultivars.

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