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Evaluating Citrus Diversity with SSR markers

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Abstract
This study evaluated the genetic diversity of nine mandarin genotypes collected from Nagpur, Amaravati, and Akola districts in Maharashtra, to aid breeding and germplasm management. Using 23 SSR markers, 21 successfully amplified loci revealed 77 alleles across 16 polymorphic markers, with an average of 3.07 alleles per primer pair. The marker CT 21 was the most informative, exhibiting the highest allele number (9) and a PIC value of 0.86. The overall average PIC of 0.58 indicated a moderate level of genetic variation among the genotypes. 'KAL-14' displayed the highest number of alleles (77), while 'N-45' had the greatest percentage of polymorphic markers (92.72%). Genetic similarity analyses using Jaccard's coefficient and a dendrogram revealed that the genotypes grouped into two main clusters based on their relationships. The SSR markers demonstrated strong discriminatory power among genotypes, underscoring their effectiveness for citrus genetic research. These insights support conservation efforts and provide a foundation for future breeding aimed at enhancing fruit quality and disease resistance.

Keywords: Mandarin, genotypes, SSR markers, Molecular characterization

Introduction
The genus *Citrus*, part of the Rutaceae family, encompasses species like *Citrus reticulata* Blanco, commonly called mandarin. This fruit is prized for its unique flavor and rich nutritional composition, including vital vitamins, minerals, and bioactive compounds that contribute to human health. Mandarins play an important role both as fresh fruit and in various food processing applications (NHB, 2017). For the effective breeding and preservation of mandarin germplasm, it is essential to accurately assess genetic diversity. Traditional morphological methods are often limited by environmental influences and the variability in physical traits, making molecular marker techniques a more reliable choice for precise genetic identification (Kalita *et al.*, 2021) [5].
Simple sequence repeat (SSR) markers are extensively utilized in genetic diversity research due to their high polymorphism, codominant inheritance, and consistent reproducibility. These markers enable the detection of allelic variations across multiple loci, making them valuable tools for genotypic identification and the management of mandarin germplasm in breeding programs (Xu *et al.*, 2013; Mullis *et al.*, 1986; Rao *et al.*, 2008) [18, 8, 9]. SSRs, also known as microsatellites, are preferred among molecular markers because of their multiallelic nature and reliability.
This study aims to assess the genetic diversity of mandarin genotypes using SSR markers, providing essential insights to support breeding efforts focused on enhancing fruit quality and promoting sustainable mandarin cultivation.

Materials and Methods
Plant Material: The study utilized nine genetically diverse mandarin genotypes, collected from various local regions of Maharashtra. The primary objective was to perform molecular characterization of this mandarin germplasm.

Molecular marker analysis
A set of 23 SSR primers, selected based on previously published studies (Fang *et al.*, 1997; Novelli *et al.*, 2004; Singh *et al.*, 2011) [4, 13], was employed for polymorphism screening and genetic diversity analysis of the mandarin genotypes.

Only those markers showing polymorphism were used for genotyping to evaluate the genetic variation within the germplasm.

DNA extraction and purification

Genomic DNA was isolated from fresh mandarin leaf tissue following a modified CTAB protocol (Doyle and Doyle, 1987) [3] with further modifications according to Cheng *et al.*, (2003) and Singh *et al.*, (2019) [2, 15]. Approximately 0.5 g of leaf tissue was ground into a fine powder under liquid nitrogen. This powder was then mixed with 1 mL of pre-warmed CTAB extraction buffer and incubated at 65°C for 1 hour, with gentle mixing every 10 minutes. Following incubation, the lysate was centrifuged at 10,000 rpm for 10 minutes at room temperature to separate debris, and the clear supernatant was transferred to fresh tubes. This supernatant underwent two rounds of extraction with phenol: chloroform: isoamyl alcohol (25:24:1), each followed by centrifugation at 10,000 rpm for 15 minutes at room temperature. The final aqueous phase was mixed with an equal volume of chilled isopropanol and incubated at −20°C for 2 hours to precipitate DNA. The DNA pellet was obtained by centrifugation at 10,000 rpm for 10 minutes at 4°C, washed with 70% ethanol, and centrifuged again at 10,000 rpm for 5 minutes at 4°C. After air-drying for 15-20 minutes, the pellet was dissolved in TE buffer and stored at −20°C. To remove RNA contamination, samples were treated with RNase A (100 µg/mL) and incubated at 37°C for 30 minutes. The quality and concentration of extracted DNA were verified by agarose gel electrophoresis and nanophotometer readings.

SSR Amplification

PCR amplification reactions were conducted in a total reaction volume of 10 µL, comprising 2 µL of genomic DNA standardized to 30 ng/µL, 0.5 µL each of forward and reverse primers at 10 pmol/µL concentration, 5 µL of commercial PCR master mix (HiMedia), and 2 µL of nuclease-free water to reach the final volume. The PCR master mix contained all necessary components for amplification, including Taq DNA polymerase, dNTPs, MgCl2, and reaction buffer, ensuring specific and reproducible PCR results. Thermal cycling conditions started with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at primer-specific optimized temperatures for 1 minute, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes (Singh *et al.*, 2019) [15]. The annealing temperature was individually optimized for each primer. PCR products were separated on a 2% agarose gel prepared in 1X TBE buffer (composed of 1 M Tris-borate and 0.5 M EDTA) containing 0.5 µg/mL ethidium bromide. Electrophoresis was run at a constant voltage of 80 V for 1.5 hours in 0.5X TBE buffer. The DNA bands were visualized under ultraviolet illumination and documented with a gel documentation system. Band sizes were estimated by comparison to a 100 bp DNA ladder.

Data scoring and genetic analysis

The PCR bands were carefully scored, with only clear and consistent bands recorded as either present (1) or absent (0), resulting in a binary data matrix for analysis. The percentage of polymorphism was determined by dividing the number of polymorphic bands by the total number of bands observed. The polymorphic information content (PIC) values for each SSR marker were calculated using the method described by Senior *et al.*, (1998) [11]. Genetic similarity between the mandarin genotypes was estimated using the Dice similarity coefficient. Based on these similarity values, a dendrogram illustrating the clustering pattern was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm implemented in NTSYS-PC software (Rohlf, 1989) [10]. To minimize bias in the analysis, null alleles were treated as missing data following the approach recommended by Warburton and Crossa (2000) [17].

Results and Discussion

The genetic diversity of nine mandarin genotypes was analyzed using 23 SSR markers, out of which 16 loci were polymorphic. These polymorphic loci amplified a total of 77 alleles, with the number of alleles per locus ranging from 1 to 9 and an average of four alleles per locus (Table 1). The overall polymorphism percentage across all loci was 49.39%. The Polymorphic Information Content (PIC) values varied from 0.20 to 0.86, demonstrating moderate informativeness of the markers and their capability to effectively discriminate among the genotypes studied (Table 1). Notably, the locus CT 21 displayed the highest number of alleles (nine) and the highest PIC value (0.86), highlighting its utility for in-depth genetic diversity evaluation.

Allelic diversity showed variation across genotypes: genotype KAL-14 exhibited the greatest allele richness with 77 alleles, whereas M-22 presented the lowest total allele count of 29. However, M-22 had a slightly higher proportion of polymorphic loci (93.10%) compared to KAL-14 (90.90%) as shown in Table 2. This suggests that even genotypes with fewer alleles can possess substantial polymorphism, which is vital for breeding program decisions. Genetic relationships among genotypes were quantified using the Dice similarity coefficient, revealing a wide range of similarity values. The closest genetic relationship was observed between genotypes K-1/03 and KAL-4 with a similarity coefficient of 0.92, while the most genetically divergent pair was KAL-19 and M-22 with a similarity of 0.30 (Table 3). Using these genetic similarity coefficients, a UPGMA dendrogram was constructed, grouping the genotypes into two major clusters with distinct subgroups. This clustering pattern reflects the underlying genetic relatedness and diversity present within the studied mandarin germplasm (Figure 2).

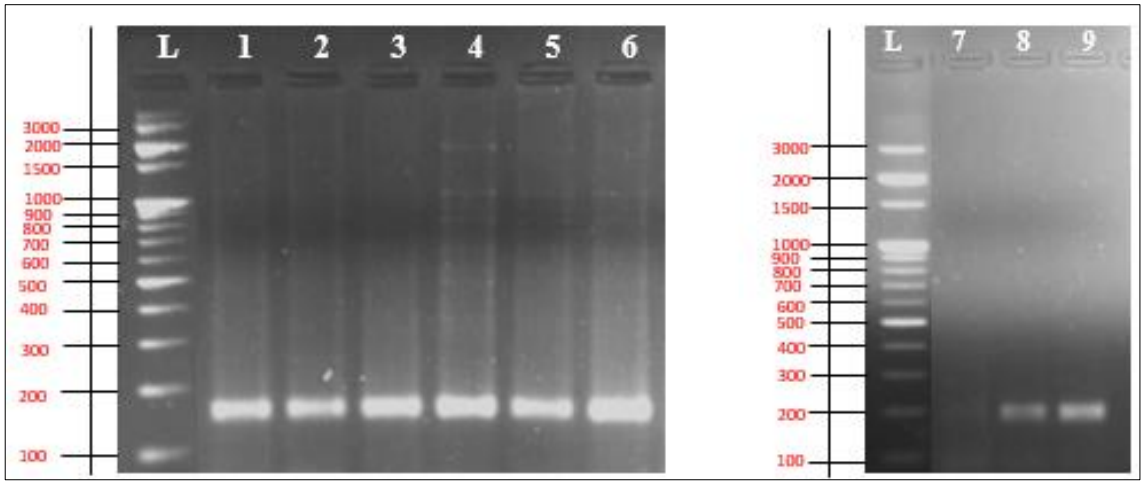

Fig 1: Allelic profiles of mandarin genotypes obtained with microsatellite marker AG14 on a 2% agarose gel. Lane L: 100 bp DNA ladder; Lane 1: K-1/03; Lane 2: N-45; Lane 3: KAL-4; Lane 4: KAL-14; Lane 5: N-16/2; Lane 6: KAL-19; Lane 7: Nagpur; Lane 8: M-22; Lane 9: ACH-37/20.

Dendrogram analysis

The dendrogram generated by UPGMA cluster analysis using SSR marker data revealed the genetic relationships among the nine mandarin genotypes, dividing them into two clearly distinct clusters with further subdivision into subgroups (Figure 2). Cluster 1 comprised three subclusters: subgroup 1A included KAL-14 and N-16/2, which exhibited very high genetic similarity, indicating close genetic affinity. Subgroup 1B consisted of K-1/03 and KAL-4, also genetically close, while subgroup 1C was formed by KAL-19, which joined this cluster at a moderate similarity level, showing its relative divergence compared to other genotypes in Cluster 1. N-45 joined this cluster at a higher similarity threshold, suggesting greater genetic differentiation within the group. Cluster 2 was further divided into two subgroups; subgroup 2A contained ACH-37/20 alone, indicating its distinct genetic identity, whereas subgroup 2B comprised M-22 and Nagpur mandarin, which clustered tightly together, indicating significant genetic similarity and possibly shared ancestry. The overall dendrogram structure highlighted Nagpur mandarin as a genetically distinct genotype relative to others in the analysis. The grouping patterns observed correspond with the Dice similarity coefficients calculated among genotypes (Table 3), which ranged from 0.30 to 0.92, reflecting a broad spectrum of genetic relationships. This hierarchical clustering effectively differentiates closely related mandarin genotypes from more divergent ones, providing critical insights for germplasm conservation and informed breeding decisions. Such clustering patterns have been similarly reported in citrus diversity studies, supporting the utility of SSR markers and dendrogram analysis for genetic relationship elucidation (Singh *et al.,* 2016) [14].

These results are in strong agreement with prior studies investigating citrus genetic diversity by SSR markers. Singh *et al.,* (2016) [14] reported comparable moderate PIC values (~0.40) and polymorphism levels between 50% and 100% among 19 mandarin genotypes from India, highlighting SSR markers' reliability for genetic variability assessments and clustering based on breeding lineage rather than geographic origin. Barkley *et al.,* (2006) [1] similarly emphasized the high informativeness of the CT 21 SSR locus, which is concordant with its prominent role in this study. Furthermore, Shahnazari *et al.,* (2022) [12] found slightly higher polymorphism (~68%) in sweet orange and mandarin populations using SSR markers, further validating the applicability and robustness of SSRs for diversity studies in citrus breeding. In summary, the moderately high percentage of polymorphic loci, informative PIC values, and well-defined clustering observed confirm substantial genetic diversity among the studied mandarin genotypes. This diversity constitutes a valuable resource for conservation, germplasm management, and breeding strategies aiming to improve citrus cultivars.

Table 1: Polymorphism (%) and PIC values of SSR markers

Sr. no.	SSR markers	Total no. of Monomorphic Band	Total no. of Polymorphic Band	No. of total Bands	Polymorphism (%)	PIC
1.	AG 14	1	3	4	75.00	0.61
2.	CAG 01	1	5	6	83.33	0.80
3.	CT 21	1	8	9	88.88	0.86
4.	CAC 33	1	3	4	75.00	0.67
5.	TAA 41	1	1	2	50.00	0.49
6.	GT 3	2	0	2	0.00	0.00
7.	CTT 1	1	3	4	75.00	0.74
8.	TAA 27	1	1	2	50.00	0.20
9.	TAA 15	3	2	5	40.00	0.79
10.	CT 2	1	5	6	83.33	0.83
11.	ATC 9	3	2	5	40.00	0.80
12.	CCT 1	1	4	5	80.00	0.80
13.	TAA 1	1	0	1	0.00	0.00
14.	TAA 52	2	2	4	50.00	0.70
15.	CAC 23	0	2	2	100.00	0.50

16.	AC 01	5	1	6	16.66	0.82
17.	TAA 45	2	0	2	0.00	0.00
18.	TAA 3	4	4	8	50.00	0.85
19.	CAC 15	1	3	4	75.00	0.75
20.	CAT 1	1	0	1	0.00	0.00
21.	CAGG 9	1	0	1	0.00	0.00
Total		34	50	84	1037.22	12.23
Mean		1.61	2.38	4	49.39	0.58

Table 2: Total number of alleles amplified in each of nine mandarin genotypes

Sr. No.	Genotypes	Number of amplified alleles		Total	Polymorphism (%)
		Monomorphic markers	Polymorphic markers		
1.	K-1/03	7	56	63	88.88
2.	N-45	4	51	55	92.72
3.	KAL-4	7	59	66	89.39
4.	KAL-14	7	70	77	90.90
5.	N-16/2	7	64	71	90.14
6.	KAL-19	7	55	62	88.70
7.	ACH -37/20	3	33	36	91.66
8.	M-22	2	27	29	93.10
9.	Nagpur mandarin	2	28	30	93.33
	Total	46	443	489	-
	Mean	5.11	49.22	54.33	90.98

Table 3: Similarity coefficient based on DNA amplification of nine mandarin genotypes estimated by dice similarity coefficient

Genotype	1	2	3	4	5	6	7	8	9
1	1								
2	0.80	1							
3	0.92	0.74	1						
4	0.80	0.71	0.79	1					
5	0.85	0.70	0.84	0.91	1				
6	0.76	0.66	0.72	0.81	0.77	1			
7	0.51	0.44	0.53	0.43	0.46	0.37	1		
8	0.38	0.48	0.40	0.36	0.36	0.30	0.73	1	
9	0.37	0.46	0.39	0.32	0.32	0.34	0.75	0.84	1

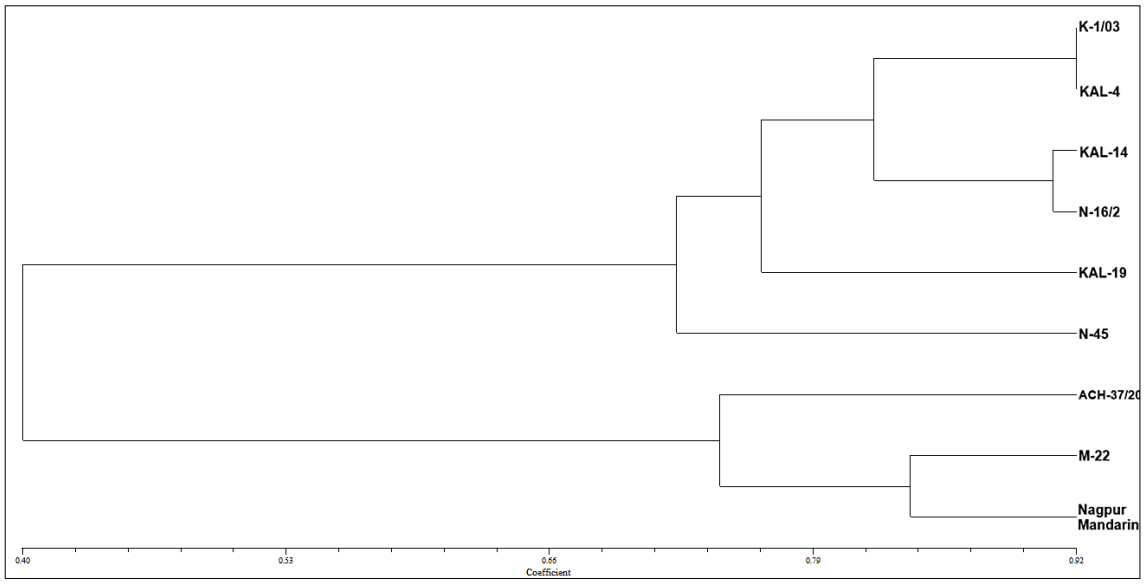

Fig 1: The dendrogram shows the genetic relationships among the mandarin genotypes based on UPGMA analysis. (*Cluster 1 is formed by three subclusters: IA includes K-1/03, KAL-4, KAL-14, and N-16/2; IB consists of KAL-19; and IC is N-45. Cluster 2 consists of 2 A with ACH-37/20, and 2 B contains M-22 and Nagpur mandarin.*)

Conclusion

The molecular analysis of mandarin accessions using SSR markers uncovered important insights into the genetic diversity within this germplasm. While the accessions showed a relatively high average genetic similarity, suggesting a likely clonal origin for mandarins in India, there remains detectable genetic variation at the molecular level. This limited but significant diversity highlights the critical need to conserve and effectively utilize the existing germplasm in breeding efforts. Additionally, the findings point to the necessity of incorporating more diverse genetic sources to broaden the genetic base. Expanding this genetic

variability is essential for the advancement of mandarin cultivars and for enhancing their tolerance to both biotic and abiotic stresses. Overall, the molecular characterization presented in this study establishes a strong foundation for future breeding and conservation strategies through informed selection and hybridization processes.

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