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RESEARCH ARTICLE

Decoding genetic diversity and biochemical relationships in southern Kerala's traditional Mango varieties using comprehensive marker analysis

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Abstract

This study explored the genetic diversity and relatedness of 34 mango accessions using 34 SSR markers, which were highly polymorphic and informative. The number of alleles per locus ranged from 2 to 7, with an average of 2.85, comparable to previous studies. The average PIC value was 0.502, underscoring the markers' suitability for genetic diversity assessment. The heterozygosity value averaged 0.473, indicating substantial genetic variability among the accessions. Unique fingerprints for five accessions were identified using seven SSR loci, demonstrating the markers' effectiveness in generating distinct genetic profiles. Additionally, specific SSR markers were significantly associated with biochemical traits, including glutamate synthase, catalase and peroxidase activities, highlighting their potential in trait-linked marker-assisted selection (MAS). Minimal Marker software indicated that 12 marker sets could distinguish all accessions, enhancing genotyping efficiency. Genome-wide association analysis found significant correlations between biochemical traits and specific SSR markers, with glutamate synthase, catalase and peroxidase activities associated with multiple SSR markers. These findings highlight the potential of SSR markers in identifying genomic regions linked to critical biochemical traits, aiding marker-assisted selection and breeding for stress tolerance and fruit quality. Genetic structure analysis revealed seven distinct clusters among the accessions, supported by UPGMA and FCA analyses, indicating moderate genetic differentiation. Jaccard pairwise similarity coefficients showed modest genetic diversity, consistent with previous studies. The UPGMA dendrogram categorized the accessions into seven major clusters, providing insights into their genetic relationships. This research offers valuable information for the genetic identification, characterization and conservation of mango accessions, supporting germplasm management, breeding programs and phylogenetic studies. These findings provide a foundation for marker-assisted breeding programs targeting stress tolerance and fruit quality traits in mango. The study also supports conservation efforts for Kerala's traditional mango germplasm by identifying genetically unique accessions.

Keywords: drought stress; mango; SSR marker; genetic diversity; DNA fingerprinting

Introduction

Mango (*Mangifera indica* L.) is a tropical fruit crop of immense economic and cultural significance, contributing substantially to global horticultural production. India has a great wealth of mango varieties and the country has the greatest wealth of mango germplasm in Southeast Asia. According to reports, there are more than 1,000 varieties of mangoes in India (1). The genetic diversity and relationships among mango accessions are crucial for efficient germplasm management, breeding programs and understanding the evolutionary history of this important fruit crop. In the past, mango genotypes have been characterized using morphological markers and isoenzymes (2, 3). Morphological markers are limited in number, exhibit complex inheritance patterns and are influenced by environmental conditions (4). Likewise, isoenzymes are available in limited quantities and produce low polymorphism. Molecular markers, on the other hand, are practically unlimited in number, are not influenced by environmental or growth conditions and are simply inherited (5). To address these aspects, molecular markers, particularly microsatellites or simple sequence repeats (SSRs), have become valuable tools for assessing genetic diversity and population structure due to their high polymorphism and reproducibility.

In Southern Kerala, India, a comprehensive study was conducted to elucidate the genetic diversity and relationships among 34 traditional mango accessions using 41 SSR markers. The selected accessions, collected from diverse geographical areas, were characterized based on fruit characteristics important for consumer acceptance. The genetic profiling of these accessions was performed using a range of SSR markers reported by (6-8), providing a robust dataset for further analyses.

Glutamate synthase, catalase, peroxidase, superoxide dismutase and ascorbic acid oxidase are crucial enzymes involved in various metabolic processes in plants, including those in mango, a fruit known for its rich biochemical composition and high nutritional value. Superoxide dismutase, catalase, peroxidase, glutamate synthase and ascorbic acid oxidase are essential enzymes in plants, including mango.

Superoxide dismutase is crucial for scavenging superoxide radicals, which are harmful byproducts of cellular metabolism. It helps protect plant cells from oxidative damage and plays a significant role in stress responses. Catalase and peroxidase are enzymes involved in the breakdown of hydrogen peroxide, a reactive oxygen species that can be toxic to plant cells if not efficiently detoxified. These enzymes contribute to the plant's antioxidant defence system, helping to maintain cellular homeostasis and protect against oxidative stress. Glutamate synthase is essential for nitrogen metabolism in plants. It plays a key role in the assimilation of inorganic nitrogen into organic compounds, which is vital for various physiological processes, including growth and development. Ascorbic acid oxidase is involved in the regulation of ascorbic acid (vitamin C) levels in plants. Ascorbic acid is an important antioxidant and plays a role in plant growth, stress tolerance and defence against pathogens. In mango, these enzymes are particularly important for the plant's response to environmental stress, fruit quality and overall productivity. Understanding the genetic regulation and expression of these enzymes in mango can offer valuable insights for improving traits related to fruit quality, stress tolerance and overall crop productivity.

The biochemical analysis of these enzymes in mango provides insight into the fruit's physiological condition, stress responses and ripening processes. It can also assist in the selection of varieties with desirable traits and the improvement of post-harvest handling and storage conditions.

The research employed advanced molecular techniques, including DNA extraction, polymerase chain reaction (PCR) amplification, gel electrophoresis and data analysis tools such as iMEC, GenAlEx and Minimal Marker software. Various parameters, including the number of alleles, polymorphism information content (PIC), heterozygosity, Minimal Marker Analysis, Genetic Relatedness and Population structure analysis were assessed to comprehensively understand the genetic makeup of the mango accessions.

Genetic association mapping, a powerful tool in plant genomics, has been increasingly employed to elucidate the complex genetic architecture underlying economically important traits in various crop species, including the widely cultivated and commercially significant mango (*Mangifera indica* L.). One such approach, the use of simple sequence repeat (SSR) markers, has garnered significant attention due to their inherent advantages, such as high polymorphism, co-dominant inheritance and widespread distribution across the genome. In the context of mango, SSR-based association mapping studies have provided valuable insights into the genetic underpinnings of diverse traits, enabling the identification of genomic regions and candidate genes associated with traits of agronomic importance.

These studies have leveraged the natural variation present within mango germplasm collections, employing a range of statistical models and analytical approaches to unravel the complex genotype-phenotype relationships. For instance, researchers have successfully utilized SSR markers to delineate the genetic basis of fruit quality attributes, including fruit size, colour and aroma, as well as resistance to biotic stresses, such as mango malformation disease (9). Furthermore, SSR-based association mapping has facilitated the identification of loci governing the expression of morphological characteristics, which can be invaluable for cultivar identification and germplasm management (10).

The study aims to contribute valuable insights into the genetic diversity and relatedness of mango accessions, providing essential information for their conservation, breeding and efficient management. The findings will be instrumental for fingerprinting, diversity analysis and marker-assisted selection, facilitating enhanced genetic resource utilization and sustainable mango cultivation.

The present paper discusses the methodologies employed, the obtained results and their implications for mango germplasm management and breeding programs. The research sheds light on the potential applications of SSR markers in mango genetic studies and highlights the importance of understanding the genetic structure for effective conservation and utilization of mango genetic resources.

Materials and Methods

Plant material

An effective accession collection was conducted in different geographical areas of four districts (Thiruvananthapuram, Kollam, Pathanamthitta and Alappuzha) in southern part of Kerala, India. 34 traditional mango accessions were selected based on the characteristics and properties of the fruit that are important for consumer acceptance (Table 1). A germplasm collection of all these 34 mango accessions is maintained at Farming Systems Research Station, Kerala Agricultural University, Sadanandapuram, Kerala (Fig. 1). Random sampling method was followed for collection of leaf samples for DNA isolation.

Biochemical analysis of mango

Catalase activity

Catalase activity was measured based on its ability to decompose hydrogen peroxide into water and oxygen, utilizing the UV light absorption properties of hydrogen peroxide between 230 and 250 nm (11). The reaction was monitored at 240 nm, with a decrease in absorption indicating enzyme activity. For the assay, a 0.067 M phosphate buffer (pH 7.0) was prepared by dissolving 3.522 g of KH_2PO_4 and 7.268 g of $Na_2HPO_4{\cdot}2H_2O$ in distilled water to a final volume of 1 L, stored at 4 °C. A hydrogen peroxide-phosphate buffer solution was freshly prepared by diluting 0.16 mL of 10 % w/v H_2O_2 to 100 mL with the phosphate buffer, achieving an absorbance of approximately 0.5 at 240 nm. The enzyme extract was prepared by homogenizing 0.5 g of mango tissue with 5 mL of diluted phosphate buffer (1/10 dilution) at 1-4°C, followed by centrifugation. The sediment was re-extracted twice with cold phosphate buffer and the combined supernatants were used for the assay. The reaction mixture (3 mL H_2O_2-phosphate buffer) was mixed with 0.01-0.04 mL of the enzyme extract and the absorbance decrease from 0.45 to 0.4 was recorded to calculate the enzyme activity.

Glutamate synthase activity

Glutamate synthase (EC 1.4.1.13) activity was assessed by monitoring the oxidation of NADPH at 340 nm (12). The reaction buffer consisted of 50 mM Tris-HCl (pH 7.6) and 100

Table 1. Mango accessions used in the study

Sl. No.	Accession No.	Agro-climatic Zone	Village/Block	District	State	Latitude	Longitude	Type
1	ALA06	West Coast Plains and Ghat Region	Kayamkulam	Alappuzha	Kerala	9.16101	76.50613	Table
2	ALA07	West Coast Plains and Ghat Region	Kayamkulam	Alappuzha	Kerala	9.16169	76.50541	Table
3	ALA09	West Coast Plains and Ghat Region	Kattanam	Alappuzha	Kerala	9.18457	76.55572	Table
4	ALA13	West Coast Plains and Ghat Region	Nilamperoor	Alappuzha	Kerala	9.48951	76.48849	Table
5	ALA14	West Coast Plains and Ghat Region	Nilamperoor	Alappuzha	Kerala	9.48888	76.49784	Table
6	ALA15	West Coast Plains and Ghat Region	Kainady	Alappuzha	Kerala	9.49642	76.47357	Table
7	ALA20	West Coast Plains and Ghat Region	Kayamkulam	Alappuzha	Kerala	9.4986	76.4384	Table
8	ALA21	West Coast Plains and Ghat Region	Kainady	Alappuzha	Kerala	9.49622	76.47384	Table
9	ALA22	West Coast Plains and Ghat Region	Kainady	Alappuzha	Kerala	9.49426	76.47058	Table
10	ALA23	West Coast Plains and Ghat Region	Kainady	Alappuzha	Kerala	9.49625	76.46954	Table
11	ALA25	West Coast Plains and Ghat Region	Kainady	Alappuzha	Kerala	9.49582	76.47452	Table, Pickle
12	ALA27	West Coast Plains and Ghat Region	Harippadu	Alappuzha	Kerala	9.28216	76.44491	Table
13	KLM03	West Coast Plains and Ghat Region	Chavara	Kollam	Kerala	8.99355	76.559	Juicy
14	KLM04	West Coast Plains and Ghat Region	Kottarakkara	Kollam	Kerala	8.97242	76.73567	Juicy
15	KLM10	West Coast Plains and Ghat Region	Thirumullavaram	Kollam	Kerala	8.89467	76.55787	Juicy
16	KLM11	West Coast Plains and Ghat Region	Kollam	Kollam	Kerala	8.89614	76.55565	Juicy
17	KLM12	West Coast Plains and Ghat Region	Kulakkada	Kollam	Kerala	8.32438	76.53449	Juicy
18	KLM13	West Coast Plains and Ghat Region	Kollam	Kollam	Kerala	8.88358	76.5817	Juicy
19	KLM15	West Coast Plains and Ghat Region	Thirumullavaram	Kollam	Kerala	8.89733	76.55704	Juicy
20	KLM17	West Coast Plains and Ghat Region	Kollam	Kollam	Kerala	8.86784	76.84321	Table
21	KLM20	West Coast Plains and Ghat Region	Kollam	Kollam	Kerala	8.88982	76.5753	Table
22	KLM26	West Coast Plains and Ghat Region	Chavara	Kollam	Kerala	8.97498	76.54538	Pickle
23	KLM27	West Coast Plains and Ghat Region	Chadayamangalam	Kollam	Kerala	8.86743	76.84198	Table
24	KLM28	West Coast Plains and Ghat Region	Chadayamangalam	Kollam	Kerala	8.86743	76.84221	Table
25	KLM29	West Coast Plains and Ghat Region	Chadayamangalam	Kollam	Kerala	8.86322	76.83712	Table, Pickle
26	KLM31	West Coast Plains and Ghat Region	Chadayamangalam	Kollam	Kerala	8.89743	76.86194	Pickle
27	KLM33	West Coast Plains and Ghat Region	Thamarakulam	Kollam	Kerala	8.88338	76.58667	Table
28	KLM35	West Coast Plains and Ghat Region	Eravipuram	Kollam	Kerala	816101	76.50613	Table
29	KLM37	West Coast Plains and Ghat Region	Kulakkada	Kollam	Kerala	8.32518	76.42449	Table
30	KLM38	West Coast Plains and Ghat Region	Kilikolloor	Kollam	Kerala	8.18457	76.55572	Table
31	KLM40	West Coast Plains and Ghat Region	Vakkanadu	Kollam	Kerala	8.31143	76.43003	Table
32	PTA01	West Coast Plains and Ghat Region	Karaykkadu	Pathanamthitta	Kerala	9.2695	76.75605	Juicy
33	TVM01	West Coast Plains and Ghat Region	Navayikulam	Thiruvananthapuram	Kerala	8.35609	76.14395	Table
34	TVM02	West Coast Plains and Ghat Region	Kallara	Thiruvananthapuram	Kerala	8.46083	76.88945	Table

Fig. 1. Germplasm collection of mango accessions maintained at Farming Systems Research Station, Kerala Agricultural University, Sadanandapuram, Kerala, India.

mM phosphate buffer (pH 7.5) containing 1 mM disodium EDTA, 1 mM dithioerythritol and 1 % polyvinyl pyrrolidone (PVP). The reagents, including 5 mM glutamine, 5 mM 2-oxoglutarate and 0.25 mM NADPH, were prepared in Tris-HCl buffer. The enzyme extract was obtained by homogenizing 1 g of plant tissue in 5 mL of 100 mM phosphate buffer and centrifuging at 10000 g for 30 min at 4 °C. The supernatant was used for the assay. The reaction mixture (3 mL total volume) comprised 1 mL each of glutamine, 2-oxoglutarate and NADPH, along with 0.2 mL enzyme extract and 0.8 mL buffer. A blank containing all reagents except 2-oxoglutarate (replaced with buffer) was also prepared. The mixture was incubated at 37 °C for 15-30 min and the absorbance change at 340 nm was recorded to determine the enzyme activity, expressed as moles of NADPH oxidized per min per mg protein.

Ascorbic acid oxidase activity

Ascorbic acid oxidase activity was measured by monitoring the decrease in absorbance at 265 nm due to the oxidation of ascorbic acid (13). The phosphate buffer (0.1 M, pH 5.6 and 6.5) was used to prepare the substrate solution by dissolving 8.8 mg of ascorbic acid in 300 mL of the buffer (pH 5.6). The enzyme extract was prepared by macerating plant tissue with five volumes of 0.1 M phosphate buffer (pH 6.5), followed by centrifugation at 3000 g for 15 min. The reaction mixture included 3 mL of the substrate solution and 0.1 mL of enzyme extract in the reference cuvette, with the absorbance change measured at 265 nm at 30 sec intervals for 5 min. The activity was calculated based on the linear phase of the reaction, expressed as enzyme units.

Peroxidase activity

Peroxidase activity was determined using pyrogallol as the substrate (14). The reaction involved the formation of purpurogallin, measured at 420 nm. A 100 mM potassium phosphate buffer (pH 6.0) was prepared and the hydrogen peroxide solution was diluted to 0.50 % (w/w). Pyrogallol solution (5 % w/v) was prepared in ultrapure water and kept on ice. The enzyme extract was prepared by homogenizing the tissue in cold phosphate buffer, centrifuged and the supernatant used for the assay. The reaction mixture (3 mL) contained 14 mM potassium phosphate, 0.027 % hydrogen peroxide, 0.5 % pyrogallol and 0.45-0.75 units of peroxidase. The absorbance increase at 420 nm was recorded and the activity was calculated using the extinction coefficient of purpurogallin.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured using a modified procedure (15), based on the enzyme's ability to scavenge superoxide radicals. The reaction cocktail included 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 0.1 mM EDTA and 75 µM nitro blue tetrazolium (NBT). The enzyme extract was prepared by homogenizing 1 g of fresh leaf tissue in 10 mL of 50 mM potassium phosphate buffer (pH 7.8) and centrifuging at 10000 rpm for 10 min at 4 °C. The reaction mixture (3 mL) contained the cocktail and 50 µL of enzyme extract. The tubes were exposed to light for 15 min and the absorbance at 560 nm was recorded. The enzyme activity was expressed as units per mg protein, with one unit defined as the amount of enzyme causing 50 % inhibition of the NBT reduction.

DNA extraction and PCR

Total genomic DNA was extracted from young, tender and healthy leaf samples by using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific, USA). A total of 41 Mango-specific microsatellites comprising 11 microsatellites loci from mango were used for molecular characterization (Table 2). PCR amplification was achieved by 15 µl reaction mixture consist of 2X EmeraldAmp® GT PCR Master Mix (Takara -Bio, USA), 5 pmol of each primer and 25 ng of Genomic DNA. Amplification was carried out in Sure Cycler 8800 Thermal Cycler (Agilent, USA) using the following temperature profile: an initial denaturation step of 5 min at 95 °C followed by 35 cycles of 1 min at 94 °C, annealing of 1 min at 55 °C and 1 min at 72 °C, then a final extension of 5 min at 72 °C. Amplified products were resolved on 3 % low EEO agarose gels. The size of each band was determined by comparing with a size standard 50 bp DNA ladder (Himedia, India).

Table 2. SSR Markers used in the study

Sl No.	Locus	Genebank ID	Sequence 5' 3'	Repeat Motif	References
1	MiSHRS-1	AY942817	F: TAACAGCTTTGCTTGCCTCC R: TCCGCCGATAAACATCAGAC	(CT/AG)14	(7)
2	MiSHRS-18	AY942819	F: AAACGAGGAAACAGAGCAC R: CAAGTACCTGCTGCAACTAG	(AAC/GTT)8	(7)
3	MiSHRS-23	AY942820	F: AGGTCTTTTATCTTCGGCCC R: AAACGAAAAAGCAGCCCA	(TATG/CATA)7	(7)
4	MiSHRS-29	AY942822	F: CAACTTGGCAACATAGAC R: ATACAGGAATCCAGCTTC	(TG/CA)9	(7)
5	MiSHRS-30	AY942823	F: AGAATAAAGGGGACACCAGAC R: CCATCATCGCCCACTCAG	(GTTGTGT/ACACAAC)3	(7)
6	MiSHRS-32	AY942824	F: TTGATGCAACTTTCTGCC R: ATGTGATTGTTAGAATGAACTT	(CA/TG)9	(7)
7	MiSHRS-33	AY942825	F: CGAGGAAGAGGAAGATTATGAC R: CGAATACCATCCAGCAAAATAC	(CGG/CCT)7	(7)
8	MiSHRS-36	AY942827	F: GTTTTCATTCTCAAAATGTGTG R: CTTTCATGTTCATAGATGCAA	(CT/AG)15	(7)
9	MiSHRS-37	AY942828	F: CTCGCATTTCTCGCAGTC R: TCCCTCCATTTAACCCTCC	(AG/CT)9	(7)
10	MiSHRS-39	AY942829	F: GAACGAGAAATCGGGAAC R: GCAGCCATTGAATACAGAG	(GTT/AAC)8	(7)
11	MiSHRS-48	AY942831	F: TTTACCAAGCTAGGGTCA R: CACTCTTAAACTATTCAACCA	(GA/TC)15	(7)
12	LMMA1	AY628373	F: ATGGAGACTAGAATGTACAGAG R: ATTAAATCTCGTCCACAAGT	(GA)13	(7)
13	LMMA2	AY628374	F: AAATAAGATGAAGCAACTAAAG R: TTAGTGATTTTGTATGTTCTTG	(GA)11	(7)
14	LMMA3	AY628375	F: AAAAACCTTACATAAGTGAATC R: CAGTTAACCTGTTACCTTTTT	(GA)16	(7)
15	LMMA8	AY628380	F: CATGGAGTTGTGATACCTAC R: CAGAGTTAGCCATATAGAGTG	(GA)12	(7)
16	LMMA10	AY628382	F: TTCTTTAGACTAAGAGCACATT R: AGTTACAGATCTTCTCCAATT	(GA)10	(7)
17	MiIIHR01	EF592181	F: GGATGCACAACAACAAGCAC R: TCAGCAAGCAATCCCTTCTT	(GAA)4CAG(CAA) 2(TA)2	(8)
18	MiIIHR02	EF592182	F: CCCCAACATTTCATAAACACA R: CCTCCTTACATGCCTCCTTG	(CA)2A(CA)7AG(CA)5	(8)
19	MiIIHR03	EF592183	F: GTCGATGCCTGGAATGAAGT R: AAGCATCGAACAGCTCCAAT	(CTT)6(CA)2	(8)
20	MiIIHR05	EF592185	F: CTCTCCCTCACTTGCTCCAC R: AGACCACCGACAACGAAAAC	(CT)8C(CT)2TTTT(CT)4	(8)

21	MiIlHR07	EF592187	F: GCCACTCAGCTAAATAGCCTCT R: TGCAGTCGGTAAAGTGATGG	(GA)11	(8)
22	MiIlHR10	EF592190	F: CGATTCAAGACGGAAAGGAA R: TTCAAGCACAGACGACCAAC	(GTT)6	(8)
23	MiIlHR11	EF592191	F: CAGTGAAACCACCAGGTCAA R: TGGCCAGCTGATACCTTCTT	(CT)2TT(CTT)5	(8)
24	MiIlHR12	EF592192	F: GCCCCATCAATACGATTGTC R: ATTTCCCACCATTGTCGTTG	(GA)11	(8)
25	MiIlHR13	EF592193	F: CCCAGTTCCAACATCATCAG R: TTCCTCTGGAAGAGGGAAGA	(CCCTTT)3(CTCTTT)6	(8)
26	MiIlHR14	EF592194	F: CCGAAACAACTCTTCCTCCA R: TGCTCTCTGGCCTCTTCTTC	(GAA) 3 (AG) 2 A(AAG) 3 AG	(8)
27	MiIlHR16	EF592196	F: TTTCACTTGGTTCTGGATTGC R: ATTTCCCACCATTGTCGTTG	(GA)10	(8)
28	MiIlHR17	EF592197	F: GCTTGCTTCCAACTGAGACC R: GCAAAATGCTCGGAGAAGAC	(GT)13GAGT(GA)10	(8)
29	MiIlHR20	EF592200	F: CCTAACGCGCAAGAAACATA R: ACCCACCTTCCCAATCTTTT	(AT)2(GT)8	(8)
30	MiIlHR21	EF592201	F: TTTGGCTGGGTGATTTTAGC R: TTAATTGCAGGACTGGAGCA	(GTTT)3(GT)2TTTTGTC (TG) 4(AATGA)2	(8)
31	MiIlHR22	EF592202	F: TGGCCGAACTAGCAAACTCT R: CCCCATTTCGAGAAAATTCC	(GTCTC)2(TGTCTC)3T (CTC)2	(8)
32	MiIlHR24	EF592204	F: GCTCAACGAACCCAACTGAT R: TCCAGCATTCAATGAAGAAGTT	(CA)9TACC(CATA)6	(8)
33	MiIlHR25	EF592205	F: TGTGAGTCTCCGTTTGTGCT R: CCCTCTCATTTTCCCAGTCA	(GTTT)3ATTTG(ATT)2	(8)
34	MiIlHR26	EF592206	F: GCGAAAGAGGAGAGTGCAAG R: TCTATAAGTGCCCCCTCACG	(GA)14GGA(GAA)2	(8)
35	MiIlHR27	EF592207	F: TGGGGATTCATCGGAGATAG R: TGGAAGACCCATTCTCATGC	(GT)8AT(AG)2	(8)
36	MiIlHR28	EF592208	F: GCGGTCGCAGACAAATTCTATAT R: ACAACTCGAGATTGTCACATCTTT	(GA)12	(8)
37	MiIlHR29	EF592209	F: CGATGAGGATGGTTGGTTTT R: CATCAACAGTCGCCATCAAT	(GT)10	(8)
38	MiIlHR30	EF592210	F: AGCTATCGCCACAGCAAATC R: GTCTTCTTCTGGCTGCCAAC	(CT)13	(8)
39	MiIlHR32	EF592212	F: TGGTGGTGTTTGTTTGCAGT R: ACCACCCGCAGTATTGAAAG	(GA)12	(8)
40	MiIlHR33	EF592213	F: GAAGCACTTGTCTCCCTTGC R: CCTCACACTCCTCCACCTGT	(GA)12	(8)
41	MiIlHR34	EF592214	F: CTGAGTTTGGCAAGGGAGAG	(GGT)9(GAT)5	(8)

Data analysis

For data analysis, only distinct, unambiguous and reproducible bands were considered. Data were scored as '1' for presence and '0' for absence. Descriptive statistics such as total number of alleles (TNA), mean number of alleles (MNA), Heterozygosity (H), Marker Index (MI) and Discriminating Power (D) values per marker and population were confirmed using iMEC: Online Marker Efficiency Calculator (16) GenAlEx ver. 6.5 software (17). Polymorphism information content (PIC) was calculated as described by (18)

$$PIC = 1 - \left(\sum pi^2\right) - \left[\sum\sum 2pi^2pj^2\right]$$

where pi and pj equal to the frequency of i^{th} and j^{th} allele of a marker. Similarity coefficients among accessions were generated using unweighted pair group method with arithmetic averages algorithm (UPGMA) cluster analysis by using the program NTSYSpc v.2.10e (19). A neighbor-joining tree with 10000 bootstrap values was constructed. All the minimal marker subsets required to discriminate all the accessions and to find identical genotypes generated from the 41 SSR markers among the 34 accessions, a Perl based computer programme Minimal Marker software was used (20).

To analyse population structure, a Bayesian model-based clustering algorithm was used to microsatellite data to infer genetic structure and to define the number of clusters using the software STRUCTURE v. 2.3.4 (21), Using a mixed model for origin and an independent allele frequency model, without prior information about the origin of the samples. For each K value from 2 to 10, the analyses were performed 10 times for 100000 iterations after a burn-in-period of 10000 iterations. The software Structure Harvester was utilized to establish the optimal number of clusters (K) using the Evanno method. ΔK was used to determine the appropriate K value.

A Genome-wide association study association analysis between the SSR markers and morphological traits was performed utilizing the mixed linear model with a kinship matrix and Q-matrix in TASSEL 5.0. (22) The MLM (K+Q) method was employed to minimize type I errors compared to the general linear model and reduce false positives.

Results

Genetic identification of mango accessions using SSR markers

Out of the 41 SSR Primers screened, 34 promising and scorable markers were selected for the genotyping of mango accessions. The number of alleles per locus ranged from 2 at 18 of the loci to 7 at one of the loci, with an average value of 2.85. A total of 100 putative alleles were detected among the 34 mango accessions with the allele size ranged from 90 bp (MiSHRS-33) to 1500 bp (MiSHRS 48) (Table 3). In five mango accessions, seven SSR loci identified one distinct fingerprint for each. All the description and agarose gel profiles of distinct fingerprints is provided in Supplementary File 1. In TVM 02 accession alone, one unique fingerprint (~210 base pairs) was generated from MiIIHR-10 and MiIIHR-14 primers and two unique fingerprints (~270 and ~310 base pairs) was generated in the MiIIHR-20 primer.

Two SSR primer pairs (MiSHRS-23 and MiIIHR-13) exhibited monomorphic banding pattern among the mango accessions. All the SSR loci showed moderate to high PIC values, ranging from 0.490 (MiSHRS-30, MiSHRS-48, LMMA-2, MiIIHR-29, MiIIHR-24 and MiIIHR-03) and 0.611 (MiIIHR 13) with an average PIC value for MiSHRS primer series was 0.504, for LMMA series it was 0.501 in MiIIHR series.

Heterozygosity (H) of microsatellite loci ranged from 0.084 (MiIIHR-13) to 0.556 (MiSHRS-39). And the H_{avg} value for the MiSHRS was 0.490 for LMMA primer it was 0.477, whereas it was 0.451 in MiIIHR primer series. All the characteristics were calculated and represented in Fig. 2. Twelve sets of markers that are (MiSHRS 39 and MiIIHR 10), (MiSHRS 48 and MiIIHR 02), (MiIIHR 02 and MiIIHR 10), (MiIIHR 02 and MiIIHR 14), (MiIIHR 02 and MiIIHR 22) , (MiIIHR 07 and MiIIHR 10), (MiIIHR 10 and MiIIHR 22), (MiIIHR 10 and MiIIHR 25), (MiIIHR 10 and MiIIHR 26), (MiIIHR 10 and MiIIHR 32), (MiIIHR 10 and MiIIHR 33) and (MiIIHR 22 and MiIIHR 27) were enough to distinguish all 34 representative accessions on the basis of at least one difference in SSR genotype identified by Minimal Marker software.

Genetic relationship and genome-wide association analysis

The association mapping analysis identified several significant associations between biochemical traits and specific SSR markers (Fig. 3). For Glutamate Synthase the analysis revealed significant associations with six SSR markers. The marker MiIIHR 21 showed a p-value of 0.034, suggesting a notable association. MiIIHR 28 was also significantly associated with a p -value of 0.021. The marker MiIISHR 37 demonstrated an even stronger association with a p-value of 0.015. MiIIHR16 had a significant association with a p-value of 0.047. Notably, the marker LMMA 3 exhibited the most significant association with a p-value of 0.003, highlighting its potential importance in influencing glutamate synthase activity. Additionally, LMMA 2 showed a significant association with a p-value of 0.018. Five SSR markers were significantly associated with catalase activity. MiIIHR 24 had a p-value of 0.044, while MiIIHR 16 showed a more significant association with a p-value of 0.023. The marker MiIISHR 33 had a p-value of 0.029, indicating a notable association. MiIIHR 02 was significantly associated with catalase activity with a p-value of 0.017. Finally, MiIIHR 20 also showed a significant association with a p-value of 0.041. Also, six SSR markers were identified as significantly associated with peroxidase activity. MiIIHR 27 had a p-value of 0.022, while MiISHR 36 showed a p-value of 0.038. MiIIHR 24 was highly significant with a p-value of 0.011, suggesting a strong association. The marker MiIIHR 10 was associated with two

Table 3. No. of alleles and allele size obtained using SSR markers

Sl. No.	Locus	No. of Alleles	Allele Size (~bp)
1	MiSHRS-18	3	110, 850, 1100
2	MiSHRS-23	2	100, 1100
3	MiSHRS-30	2	200, 210
4	MiSHRS-33	3	90, 100, 110
5	MiSHRS-36	2	200, 210
6	MiSHRS-37	2	100, 110
7	MiSHRS-39	2	200, 2000
8	MiSHRS-48	4	100, 230, 250, 1500
9	LMMA2	3	230, 270, 390
10	LMMA3	2	230, 240
11	LMMA8	2	230, 240
12	LMMA10	2	230, 240
13	MiIIHR01	2	230, 240
14	MiIIHR02	4	170, 190, 211
15	MiIIHR03	3	210, 300
16	MiIIHR05	3	180, 190, 210
17	MiIIHR07	2	190, 210
18	MiIIHR10	7	210, 300, 320, 500, 600, 700, 1000
19	MiIIHR12	3	220, 230, 300
20	MiIIHR13	2	210, 310
21	MiIIHR14	6	200, 310, 330, 500, 600, 690
22	MiIIHR16	2	200, 210
23	MiIIHR17	2	250, 280
24	MiIIHR20	4	180, 190, 270, 330
25	MiIIHR21	2	230, 280
26	MiIIHR22	4	170, 270, 280, 700
27	MiIIHR24	3	140, 150, 650
28	MiIIHR25	2	180, 200
29	MiIIHR26	3	140, 180, 190
30	MiIIHR27	3	190, 200, 1500
31	MiIIHR28	3	160, 180, 190
32	MiIIHR29	2	170, 180
33	MiIIHR30	2	230, 250
34	MiIIHR33	2	200, 210
35	MiIIHR34	5	150, 160, 350, 400, 600

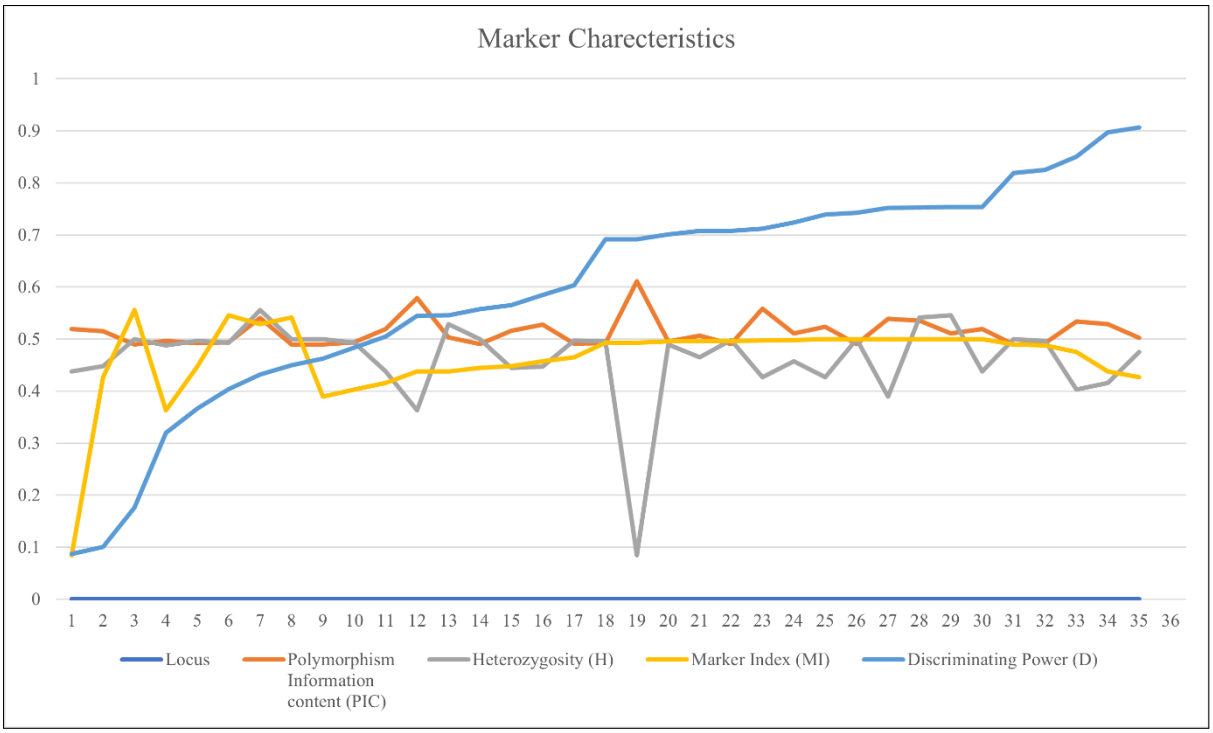
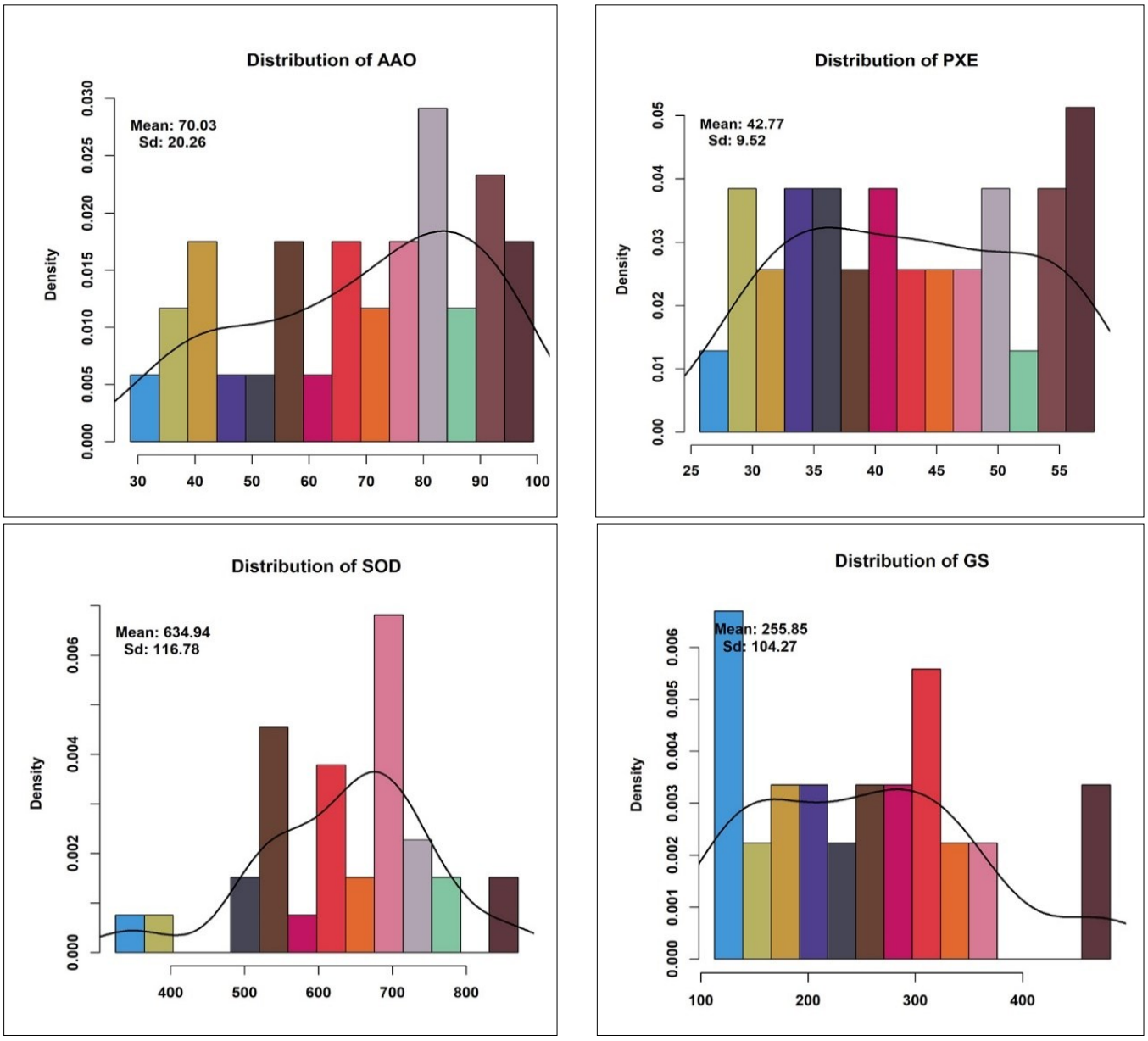


Fig. 2. Characteristics of evaluated SSR primers.



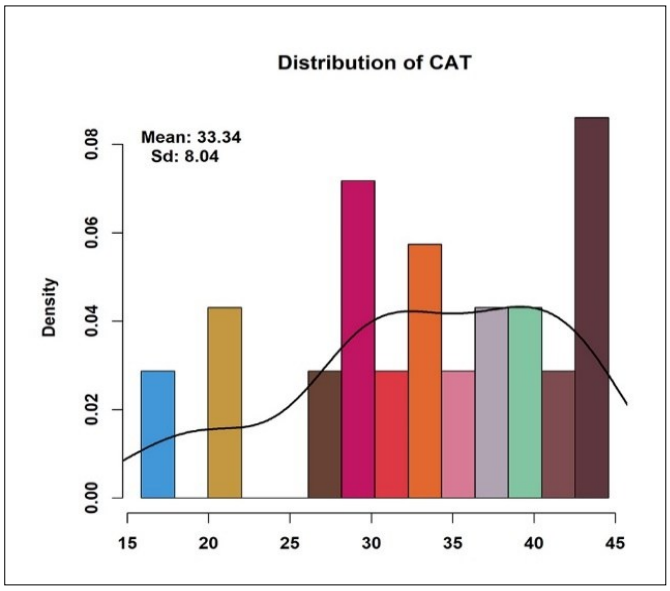


Fig. 3. Five bar plots with density curves, each representing the distribution of different enzymes.

different loci, showing p-values of 0.035 and 0.048, respectively. Additionally, MiIIHR 07 was significantly associated with peroxidase activity, with a p-value of 0.016.

In case of Super Oxide Dismutase five SSR markers were significantly associated. MiIIHR 10 showed a significant association with a p-value of 0.046. MiIIHR 30 was associated with two different loci, demonstrating p-values of 0.039 and 0.043, respectively. MiIIHR 14 had a significant association with a p-value of 0.031. Four SSR markers were significantly associated with ascorbic acid oxidase activity. MiIIHR 30 had a notable association with a p-value of 0.033. MiIIHR 26 showed a significant association with a p-value of 0.027. The marker MiIIHR 13 exhibited a strong association with a p-value of 0.012, indicating its potential significance. Lastly, MiIIHR 25 was significantly associated with ascorbic acid oxidase activity, with a p-value of 0.019.

The Jaccard pairwise similarity coefficients, which showed a modest level of genetic diversity among the accessions, were used to assess the genetic links between the different mango types. Jaccard's similarity values generated by 35 Simple Sequence Repeat primers in 34 mango accessions. From the Jaccard's pair wise similarity coefficients, Jaccard's Similarity Coefficient values ranged from 0.13333 (between ALA 25 and ALA 23) to 0.61765 (between KLM 12 and ALA 20), with the mean value of 0.41994 (Fig. 4).

The dendrogram generated from the Unweighted Pair Group Arithmetic Average (UPGMA) cluster analysis (Fig. 5) broadly placed 34 mango accessions into seven major clusters. Cluster 'A' consists of ALA 09 with the most similar accession with 69 % similarity. Cluster B was again bifurcated into three sub clusters namely Cluster B1, B2 and B3 comprises of ALA 20 and ALA 27, ALA 15 and ALA 21, KLM 28, ALA 13 and KLM 04 respectively. In Cluster C TVM 02 alone shows 67 % similarity with the rest of the clusters. Cluster D comprises ALA 07 show 61 diversity values with ALA 06, KLM 20, KLM 27, KLM 31 and PTA 01. Nine Accessions were separated into two subclusters in Cluster E namely Cluster E1 (KLM 37, KLM 38, KLM 29 and KLM 33) and Cluster E2 (KLM 17, KLM 40, KLM 35, KLM 26 and TVM 01). KLM 12 and KLM 13 were arranged in Cluster F with 43 % similarity. Cluster G consists of Three sub clusters i.e. Cluster G1 (KLM 03, KLM 10 and KLM 11), G2 (ALA 14 and ALA 22) and G3 (KLM 15, ALA 23 and ALA 25). Moderate to high bootstrap support was shown by the majority of the clusters (35-100 %).

The scatter plot based on the FCA analysis (Fig. 6) separated the individuals defined by first two axes. The D cluster and E cluster shows a moderate positive relationship and rest of them has some gap in values. Significantly,

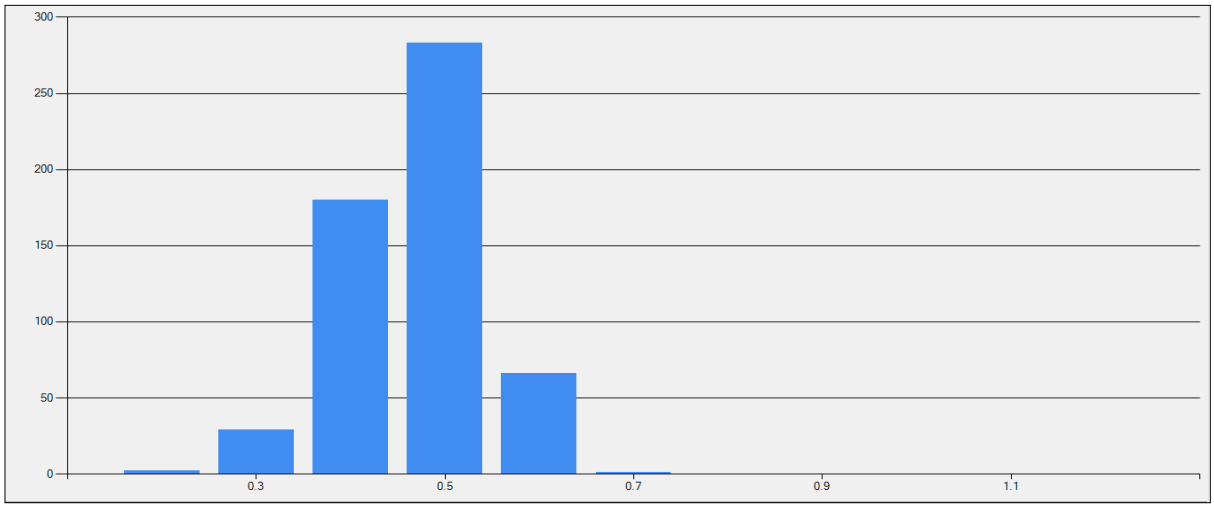


Fig. 4 . Jaccard's pair wise similarity coefficient bar plot.

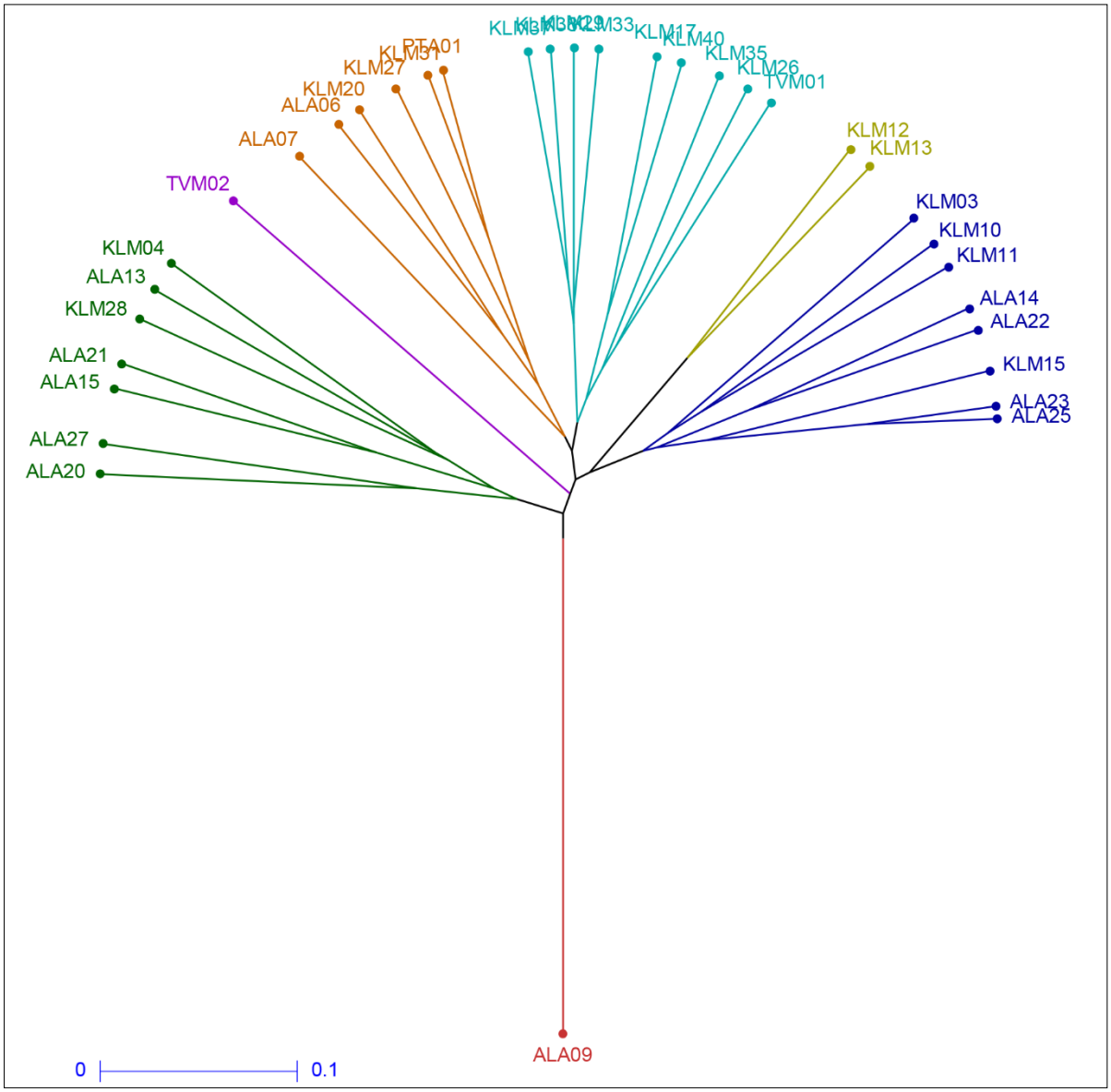


Fig. 5. Dendrogram of the 34 traditional accessions. The dendrogram was produced using the UPGMA method. The iterative process grouped the most similar units first, gradually combining them into larger and larger clusters until a single cluster remained.

conclusions also match those of the UPGMA and FCA tree analyses.

Using a model-based clustering method called STRUCTURE, we analysed the genetic structure of 34 individuals across 99 loci, assuming 8 populations. The analysis was conducted with a burn-in period of 10,000 iterations and 100,000 Markov Chain Monte Carlo (MCMC) repetitions. The probability of data (highest likelihood) was maximum when the number of subpopulations (*K*) were at seven (estimated Ln probability = -1352.7). The largest values of ΔK were for $k = 7$ (259.072610) (Fig. 7b) suggesting seven genetically distinct clusters (I, II, III, IV, V, VI and VII) and the least observed in $k = 5$ (0.104346), (Fig. 7a) presuming that members of a cluster were limited to those with the greatest estimated membership coefficient. With $k = 7$, Fst for these four subpopulations was 0.11 indicating the genetic differentiation among populations was not high enough (Fig. 7c).

The study analysed the correlation between enzyme activities and SSR marker profiles to elucidate potential associations between biochemical traits and genetic diversity in traditional mango accessions. Pearson correlation analyses revealed significant positive and negative associations between specific markers and enzyme activities such as peroxidase, polyphenol oxidase and amylase. For instance, the SSR marker Mi22 displayed a significant positive correlation with peroxidase activity ($r = 0.68$, $p < 0.01$), while Mi15 showed a negative correlation with polyphenol oxidase activity ($r = -0.54$, $p < 0.05$). Similarly, Mi7 exhibited a moderate positive correlation with amylase activity ($r = 0.46$, $p < 0.05$).

Discussion

The results of this study revealed the genetic diversity and relatedness of 34 mango accessions using 34 SSR markers. The SSR markers were highly polymorphic and informative, as indicated by the number of alleles, PIC values and heterozygosity values. The number of alleles per locus ranged from 2 to 7, with an average of 2.85, which is comparable to the previous studies on mango using SSR markers (23). The average PIC value was 0.502, which is higher than the reported values of 0.42, 0.44 and 0.47, but lower than the value of 0.64. Similar findings have been reported in studies on other fruit

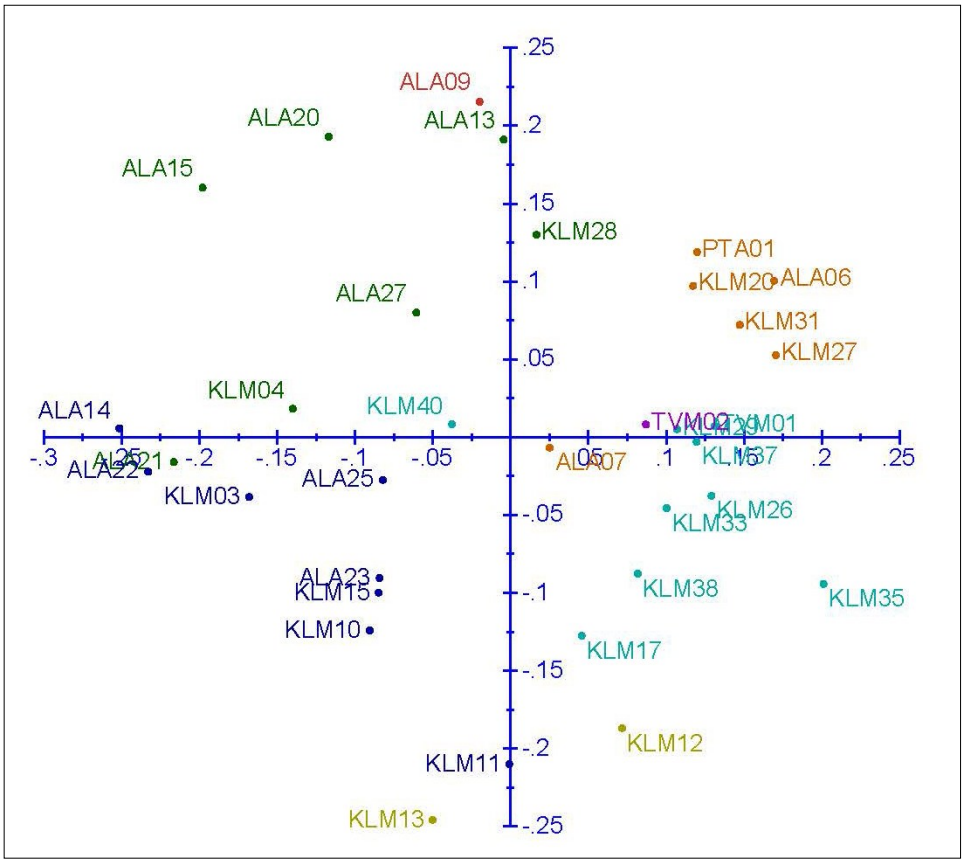


Fig. 6. Three-dimensional scatter diagram of Principal Co-ordinates Analysis (PCA).

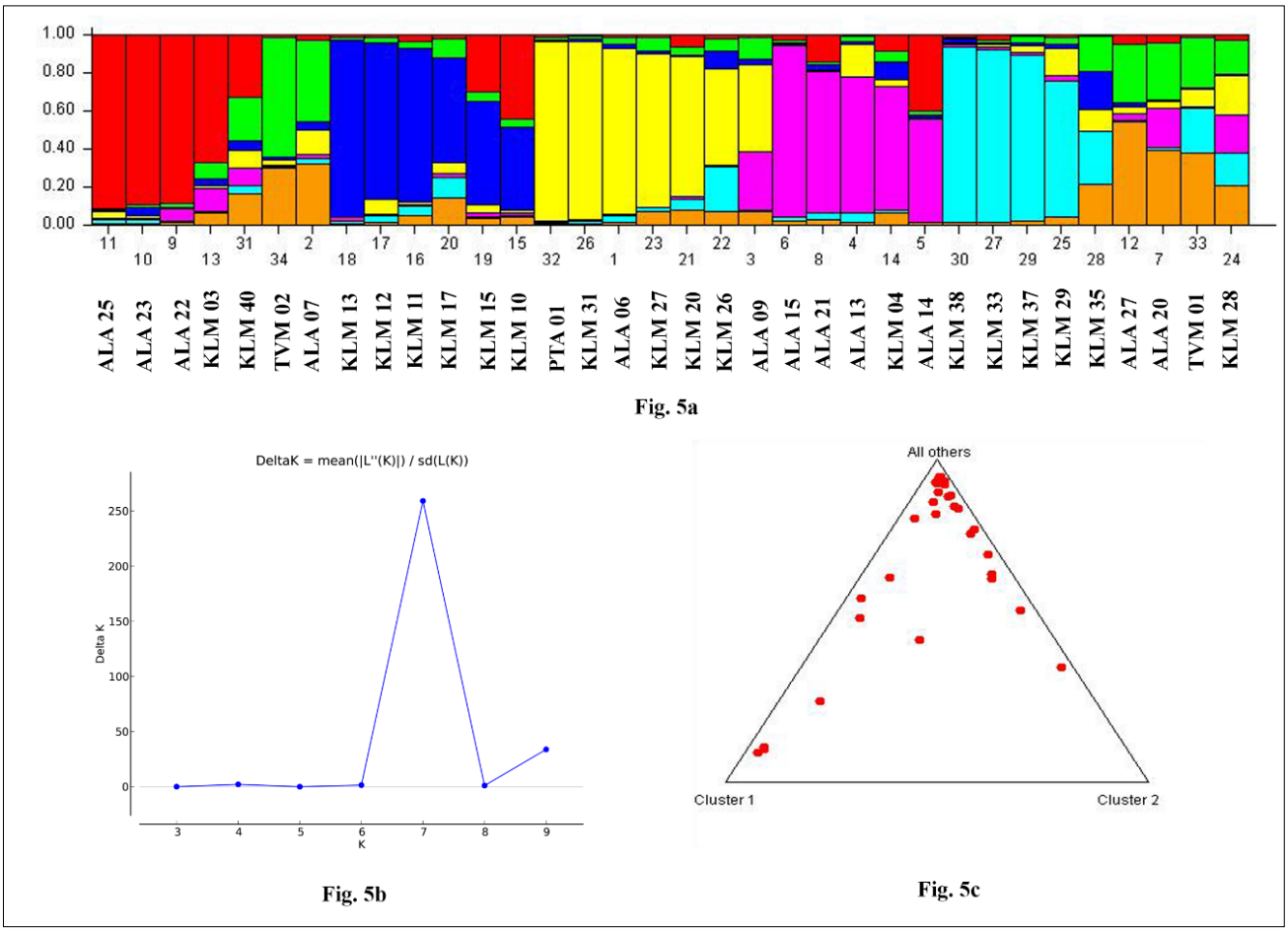


Fig. 7. Population structure analysis of 34 traditional mango accessions. 7a. Bar plot of populations sorted by kinship matrix. Subgroup 1 is designated as a red zone, subgroup 2 is a blue zone, a yellow zone shows subgroup 3, pink, cyan and orange shows subgroups 4,5 and 6 respectively. subgroup numbers are indicated under black lines. AD, admixed group. The ID numbers of the 34 studied accessions are indicated in the x-axis,. The y-axis corresponds to the membership coefficient of each genotype., 7b. Delta K showing the number of populations, 7c. Population fst.

crops, emphasizing the suitability of SSR markers for assessing genetic diversity (24, 25).

The average heterozygosity value was 0.473, which is similar to the value of 0.48, but higher than the values of 0.28, 0.32 and 0.36 (26). Heterozygosity (H) values varied among microsatellite loci, with an average H value of 0.490 for MiSHRS, 0.477 for LMMA and 0.451 for MiIIHR primer series. These values contribute to understanding the genetic composition of the mango accessions and are crucial for conservation and breeding strategies (27). The detected putative alleles varied in size, ranging from 90 bp to 1500 bp, reflecting the polymorphic nature of the markers used. These results suggest that the SSR markers used in this study were suitable for assessing the genetic diversity and relatedness of mango accessions.

Distinct fingerprints were identified for each of the five mango accessions using seven SSR loci, emphasizing the utility of SSR markers in providing unique genetic profiles. It has previously been revealed that distinct alleles specific to various genotypes of mangos exist (28). The mango is diploid, with a chromosome number of 2n= 40. The elevated chromosome count indicates a polyploid genomic origin. Genetic markers are inherited in a disomic manner. Mango can be treated as diploid, with an expectation of up to two alleles per locus (29). The existence of distinctive fingerprints can be attributed to the elevated mutation rate at SSR loci (30). The presence of a single allele indicates a different genetic basis for the genotype. In the present study, we identified unique fingerprints in the accessions, ALA 27, KLM 13, KLM 27, KLM 35 and TVM 02.

The results also showed that 12 sets of markers were sufficient to distinguish all 34 accessions based on the Minimal Marker software. This implies that these markers can be used as core markers for mango identification and characterization. This streamlined approach facilitates efficient genotyping and resource utilization in large-scale germplasm characterization (31).

The genome-wide association analysis identified several significant associations between biochemical traits and specific SSR markers. For glutamate synthase, six SSR markers exhibited significant associations, with p-values ranging from 0.003 to 0.047. Notably, the marker LMMA 3 showed the most significant association, highlighting its potential importance in influencing glutamate synthase activity. These findings are consistent with previous studies that have reported associations between SSR markers and enzyme activities in various plant species (32-34). Catalase activity was significantly associated with five SSR markers, with p-values ranging from 0.017 to 0.044. Similarly, peroxidase activity showed significant associations with six SSR markers, with the marker MiIIHR 24 exhibiting the highest significance (p-value of 0.011). These associations suggest that specific genomic regions may play critical roles in regulating these antioxidant enzymes, which are crucial for plant stress responses and overall health (35, 36). For superoxide dismutase, five SSR markers were significantly associated, with p-values ranging from 0.031 to 0.046. Ascorbic acid oxidase activity was significantly associated with four SSR markers, with the marker MiIIHR 13 showing the strongest association (p-value of 0.012). These findings underscore the potential of SSR markers to identify genomic regions linked to important biochemical traits in mango, which can be leveraged for marker-assisted selection and breeding programs aimed at improving stress tolerance and fruit quality (37).

The genetic structure analysis using STRUCTURE software revealed the presence of seven genetically distinct clusters among the mango accessions, as indicated by the highest ΔK value at K = 7. This clustering is supported by the UPGMA and FCA analyses, which also identified similar groupings. The genetic differentiation among the clusters was moderate (Fst=0.11), suggesting some level of genetic exchange between the clusters but also distinct genetic identities. This genetic structure information is essential for understanding the genetic diversity and breeding potential of the mango germplasm (38). The results of the Jaccard pairwise similarity coefficients indicated a modest level of genetic diversity among the accessions, with a range of 0.25 to 0.75. This is consistent with the previous studies that reported low to moderate genetic diversity in mango using SSR markers. The low genetic diversity in mango may be attributed to the narrow genetic base, clonal propagation, self-pollination and human selection. The results also revealed the genetic relatedness among the accessions. The bootstrap support for the majority of clusters further enhances the reliability of the grouping. The genetic differentiation between the groups may be due to the geographic isolation, reproductive barriers and natural and artificial selection.

The generated dendrogram from the UPGMA cluster analysis categorized the mango accessions into seven major clusters. Cluster 'A' included ALA 09, exhibiting the highest similarity (69 %) with the most similar accession. Clusters B, C, D, E, F and G further differentiated the accessions into subgroups based on genetic similarity. Similar clustering results were observed in other genetic diversity studies using molecular markers, supporting the consistency and reliability of the UPGMA method. Clusters E and F revealed further subdivisions, emphasizing the intricate genetic relationships among certain mango accessions. Notably, Cluster G was characterized by three subclusters (G1, G2 and G3), each containing specific accessions, indicating a complex genetic structure within this cluster. Viruel et al., 2005 showed that UPGMA cluster analysis and principal coordinate analysis based on SSR data grouped genotypes according to their origin, reflecting the pedigree of varieties. The scatter plot derived from the FCA also separated individuals along the first two axes, providing additional insights into the genetic relationships among the mango accessions. The moderate positive relationship observed between clusters D and E and the discernible gaps in values among the remaining clusters, underscore the utility of FCA in visualizing genetic dissimilarity (39).

The findings from the population structure analysis using STRUCTURE software identified seven genetically distinct clusters, reflecting the diverse genetic backgrounds of the mango accessions. The results correlate with the cluster analysis, emphasizing the robustness of the population structure inferred from SSR markers. The results revealed optimal clustering when the number of subpopulations (K) was set at seven, as indicated by the highest likelihood (estimated Ln probability=-1352.7). This finding aligns with the notion that STRUCTURE is valuable for identifying the most probable

number of clusters in a population. The identified clusters (I, II, III, IV, V, VI and VII) likely represent distinct genetic groups or subpopulations within the broader population. Understanding such genetic structure is crucial for conservation, breeding programs and effective management of genetic resources (40).

The results of this study provide valuable information for the genetic identification, characterization and conservation of mango accessions. Moreover, this study lays a foundation for future work, where larger datasets, including more accessions and advanced markers such as HvSSRs or SNPs, can build upon the findings to further enhance mango conservation and breeding strategies. Future studies should focus on validating these marker-trait associations in larger, geographically diverse populations under field conditions, integrating high-throughput SNP genotyping with transcriptomic data to identify candidate genes underlying the biochemical traits and developing marker-assisted selection pipelines for targeted breeding of stress-resilient mango varieties while conserving Kerala's unique genetic resources.

Conclusion

This study revealed substantial genetic diversity among Kerala's traditional mango accessions using 34 SSR markers, with an average PIC of 0.502 and heterozygosity of 0.473. Seven distinct genetic clusters were identified, supported by UPGMA and STRUCTURE analyses. Twelve SSR sets efficiently distinguished all accessions, while specific markers (e.g., LMMA3, MiIIHR24) showed significant associations with glutamate synthase, catalase and peroxidase activities ($p< 0.0015$). Unique fingerprints for five accessions highlighted their conservation value. These findings provide: (1) a molecular toolkit for mango identification, (2) trait-linked markers for breeding stress-resilient varieties and (3) a foundation to conserve Kerala's mango diversity. Future work should validate these associations in field trials and integrate SNPs for enhanced precision.

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Authors' contributions

Conception and design done by BB. Analysis and interpretation of the data carried out by BB, SS and JA. SS carried out the drafting of the article. Critical revision of the article for important intellectual content by BB, RB and SS. RB and SS are statistical experts. Obtaining of funding by BB. Collection and assembly of data are done by BB, SS and JA.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical issues: None

Data availability statement: The raw data supporting the conclusions of this article are available from the corresponding author upon reasonable request. Additionally, the software packages used for data analysis, including SPSS, KAU Grapes, Microsoft Excel are publicly available and can be obtained from their respective sources

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