Brief Report

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Brief Report

SSR Markers-Based DNA Fingerprinting for Varietal Identification in Mango Cultivars

Muhammad Aleem Ashraf [1,2,3,†,*], Javed Iqbal [4,†], Imran Shahid [5,†], Muhammad Kashif [6], Yufeng Li [1], Yu Kong [1] and Naitong Yu [1,3,*]

[1] Guangxi Key Laboratory of Biology for Mango, College of Agriculture and Food Engineering, Baise University, Baise 533000, China; 563728028@qq.com (Y.L); 190236181@qq.com (Y.K)
[2] Department of Biosciences and Technology, Emerson University, Multan 60000, Pakistan
[3] Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, 571101, China
[4] Mango Research Station, 6-Km Basti Malook Road, Shujabad 599220, Pakistan; javedsaqi@gmail.com
[5] Department of Pharmacology and Toxicology, Faculty of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia; iyshahid@uqu.edu.sa
[6] Department of Chemistry, Emerson University, Multan 60000, Pakistan; muhammad.kashif@eum.edu.pk
* Correspondence: aleem.ashraf@eum.edu.pk (M.A.A.); yunaitong@163.com (N.Y.)
† These authors contributed equally to this work.

Abstract: Mango (*Mangifera indica* L.) is an allotetraploid (2n = 4X= 40) drupe fruit and has high nutritional value belongs to genus Mangifera and family Anacardiaceae. Mango cultivars are used with worldwide acceptance to pharmacological, ethnomedical, and phytochemical industries. Assessment of the genetic distinctiveness of a cultivar through morphological descriptors is an important tool for both the registration and the protection. New mango genotypes have been improved using valuable diverse germplasm resources to ensure food security. DNA fingerprinting based simple sequence repeats (SSR)-markers have been the most broadly used, effective and accurate in evaluation of genetic characterization of a cultivar. Molecular breeding is an effective source of genetic gain after improvement of fruit trees using marker assisted genomic selection. Total genomic DNA (gDNA) was generated using CTAB method from each cultivar. The most effective 50 hyper-variable SSR markers were selected. Highly specific DNA fingerprints were identified in the candidate line 'Azeem Chaunsa' compared with three standard cultivars using SSR-PCR. An agglomerative hierarchical clustering method was used to construct dendrogram based on the UPGMA clustering method. Cultivar identification diagram (CID) was constructed to evaluate association among standard cultivars and Azeem Chaunsa. Our results showed that SSR markers could efficiently assess genetic diversity in mango. The genetic similarity coefficients were recorded between the cultivars of mango ranged from 0.49 to 0.67. CID results concluded that cultivar 'Azeem Chaunsa' varied significantly from the check cultivar, Sindhri (46.2%), S.B Chaunsa (45%) and Sufaid Chaunsa (46.7%). The results obtained in this study will orient cultivar identification strategies for a successful future.

Keywords: mango tree; SSR markers; mango varieties; genetic diversity; cultivar identification diagram, loci, polymorphism; genetic resources in Pakistan; breeding; cultivar development

1. Introduction

The mango (*Mangifera indica* L.) is the most popular cultivated commercial fruit with great economic value and important long-lived evergreen tree [1]. Mango has been ranked third in production and second major fruit in Pakistan, participating to food and nutritional security to rural economy. Mango is known as "king of fruits" originated from subcontinent belonging to an earliest cultivated fruits. Mango production is concentrated in Pakistan in Sindh province as tropical climate

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while in Punjab have the subtropical climate [2]. Pakistan has commercial exportable mango cultivars as Sindhri (Early), SB Chaunsa (Mid) and Sufaid Chaunsa (Late) fruit availability and have approximately 3.5 months window for export of mangoes to other countries [3]. Mango fruit trees have been widely grown in tropical and subtropical areas. China and Pakistan are among the top mango producer countries. Mangoes are one of the major horticultural fruit crops in Punjab with 76% and Sindh provinces 24% share in mango production in Pakistan. In China, tropical provinces such as Hainan, Guangxi, Guangdong, Yunnan, Fujian, and Sichuan are major mango producers. Selection among chance seedling with superior traits of fruit quality as well as planned breeding with marker assisted selection considered a quick and precise technique for the development of mango varieties. Mango is often cross-pollinated [4], an allotetraploid (2n = 4X= 40) [5], highly heterozygous tree fruit, mono- and polyembryonic seed and having small imputed genome size of approximately 440-480 MB[6,7]. The genes controlling the traits can be mapped using high-throughput sequencing [8,9]. New mango varieties are generated by hybridization, introduction, selection and breeding of novel transgenes or genotypes [10,11].

Commercial mango varieties have largely been grown under varying planting geometries and were maintained by using clonal propagation by grafting of a specific variety or mutated branches [12]. Mango trees are heteroecious and cross-breeding has become dominant with high world popularity. Simple sequence repeats (SSR) or microsatellites are categorized as genetic loci and are tandem repeats, highly abundant and broadly distributed across both the prokaryotic and eukaryotic genomes. Compared with RFLP and RAPD, the advantages of SSR molecular markers include multi-allelic, clear loci, highly polymorphic, good repeatability, high resolution, codominate, reliable detection, high abundance, simple experimental design, easy operation and high distribution in plant genomes. The SSR DNA markers are widely used for genotype DNA identification, variety or hybrid certification, parent detection, diversity analysis in diverse forest fruit species including mango [13–15]. More than 1000 named mango commercial cultivars have been reported to exist around the worldwide. More than 100 SSR markers have been used to identify, characterize and evaluate various mango germplasm [16–18]. Germplasm evaluation and genetic diversity in mango using SSR markers gain significant advancement for evaluation of hybrids or cultivars, determination of genetic variations and conservation of germplasm [7]. SSR markers have widely used for identification of the domestication and movement of germplasm [19]. Genetic diversity evaluation in candidate cultivar using SSR molecular technology is based on SSR-PCR amplifications. The polyacrylamide gel electrophoresis and silver staining procedures were used to visualize and analyze the amplified segments as DNA fingerprints [20,21].

In Pakistan, mango is an exportable fresh fruit commodity. Several breeding lines are created to develop competitive cultivars with excellent production [22]. In mango, variety evaluation is important for utilization of the valuable genetic resource. The present study was performed to estimate the genetic variability created in mango cultivar ‘Azeem Chaunsa’ recommended for cultivation in Punjab using SSR markers. The genetic diversity of Azeem Chaunsa’ cultivar was also compared with other improved standard mango cultivars cultivated in Punjab. Mango reciprocal cross breeding method is frequently used for breeding new mango cultivars in hybridization program [23]. The progeny have the probability of both superior and inferior traits, tested after passing long juvenility. It is difficult to differentiate the authenticity of the offspring of hybrid. To evaluate cultivar identification and diversity of Pakistani mango genetic resources, 50 standard polymorphic SSR markers were selected for rapid genetic purity assessment in mango. Several commercially grown mango cultivars or hybrids were assessed accurate parentages. The present study was performed to determine genetic diversity among candidate mango cultivar and standard mango cultivars using hyper-variable polymorphic SSR markers. In the current study, unique and rare alleles were also identified and reconfirmed that would be useful for determination of genetic purity of cultivars in mango.

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2. Materials and Methods

2.1. Experimental Material

The mango cultivars classified according to fruit availability such as Sindhri (Early season), Samar Bahisht (S.B) Chaunsa (Mid-season) and Sufaid Chaunsa (Late season), and the candidate cultivars 'Azeem Chaunsa' were grown in separate block and follow the planting geometry of 27 feet distance in between the rows and 22 feet distance in plants which accommodated 72 plants per acre in Mango Research Station, Shujabad, Multan. Freshly emerged tender leaves were collected for extraction of DNA samples. A set of perfect mango polymorphic SSR markers was selected for testing based on high polymorphism, stable amplification and clear banding patterns. The SSR primers were obtained from different genomic databases based on wide genome coverage. The fully grown mature and uniform mango trees having uniform age and size in the experimental orchard of "Mango Research Station" Shujabad, Punjab (Pakistan), located at latitude 29.8717° N and 71.3231° E, belonging to the Sub-Tropical Arid Climate. The standard cultivars are commercially grown in all the provinces of Pakistan and differ in geographical region. The standard cultivars such as Sindhri, Samar Bahisht (S.B) Chaunsa and Sufaid Chaunsa, have been approved by Punjab Seed Council, Lahore, Punjab, Pakistan. Experimental materials were collected in compliance with the institutional, national, and international guidelines and legislation.

2.2. Genomic DNA (gDNA) Extraction and Analysis

Total genomic DNA (gDNA) was generated from 4-5 young fully expanding leaves of each cultivar. The gDNA extraction was performed using the dried ground leaves of seedlings using the cetyltrimethylammonium bromide (CTAB) protocol with minor modification. The quality of gDNA was evaluated by loading 15 ng DNA of each genotype on 0.8% agarose gel prepared in IX TBE buffer and stained with ethidium bromide (10 ng/100 ml). Samples showing intact bands were selected to use for further study. The DNA concentration and purity of each cultivar was determined using a Nano Drop® ND-1000 spectrophotometer by estimating absorbance ($OD_{260/280}$). Intact gDNA bands were marked for further SSR-PCR. The gDNA was stored at − 20 °C.

2.3. Search for Mango Simple Sequence Repeat Markers and Choice of PCR Primers

SSR markers were selected from the reference databases [18,24–26]. A total of 50 pairs of highly polymorphic SSR primers with different amplification bands among 'Sufaid Chaunsa', 'Sindhri', and S.B. Chaunsa 'and' Azeem Chaunsa' were selected for cultivar identification (**Table 1**). The amplification efficiency of the selected SSR markers was evaluated using SSR-PCR.

Table 1. List of microsatellite markers used in mango DNA fingerprinting study.

SSR Primer Pair ID	Forward Primer	Reverse Primer
LMMA1F	ATGGAGACTAGAATGTACAGAG	ATTAAATCTCGTCCACAAGT
LMMA7F	ATTTAACTCTTCAACTTTCAAC	AGATTTAGTTTTGATTATGGAG
LMMA9F	TTGCAACTGATAACAAATATAG	TTCACATGACAGATATACACTT
mMiCIRO14	GAGGA CATAAAGATGGTG	GACAAGATAAACAAC TGGAA
mMiCIRO18	CCTCAATCTCACTCAACA	ACCCCACAATCAAACTAC
mMiCIRO32	TCATTGCTGTCCCTTTTC	ATCGCTCAAACAATCC
MiSHRS-1	TAACAGCTTTGCTTGCCTCC	TCCGCCGATAAACATCAGACA
MiSHRS-48	TTTACCAAGCTAGGGTCA	CACTCTTAAACTATTCAACCA
MIAC-4	CGTCATCCTTTACAGCGAACT	CATCTTTGATCATCCGAAAC
MIAC-6	CGCTCTGTGAGAATCAAATGGT	GGACTCTTATTAGCCAATGGGAG
MGDSSR1	CGAAATGAGACACCTGCAAA	TTTCCTCCATTGCTTTTTCG
MGDSSR2	GGGAATGGTAGAGACGGACA	ATCCAAGCAGTCACCATCAA
MGDSSR5	CGATAGTGCCAATCTGGTGA	TCATCTCACACACTCTCTCTCTCTC
MGDSSR11	GGGAATGGTAGAGACGGACA	TTCATCATAGGTCCCACACG
MGDSSR14	AATGCTGAGCCTGGTAAGGA	CAACATCCTCTTTCTTCCCTGT

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MGDSSR34	GAAAGTGAGACCTTCGGTTCC	AAGGCCCCTTCTTCACATTT
MillHR21	TTTGGCTGGGTGATTTTAGC	TTAATTGCAGGACTGGAGCA
mMiCIR005	GCCCTTGCATAAGTTG	TAAGTGATGCTGCTGGT
mMiCIR009	AAAGATAAGATTGGGAAGAG	CGTAAGAAGAGCAAAGGT
mMiCIR013	GCGTAAAGCTGTTGACTA	TCATCTCCCTCAGAACA
mMiCIR016	TAGCTGTTTTGGCCTT	ATGTGGTTTGTTGCTTC
mMiCIR030	GCTCTTTCCTTGACCTT	TCAAAATCGTGTCATTTC
MiSHRS-37	CTCGCATTTCTCGCAGTC	TCCCTCCATTTAACCCTCC
MIAC-11	GTGCGAGGAGATATCTGT	CTGGTTCTTCATTGTTGAGATG
MITGI75	TGCGTCTTGTGTGTGTGTGT	GGAATGCTGTGTGTGTGTG
MITGg62	TGTTCGATTTGCAAACTTTTT	GGCCTAATGTGTGTGTGTG
MICA231-1	TGGAAGGACCATGCTTGAAT	GGTCACACACACACACACA
MICA235	TGTCACACACACACACACA	AATGGAAGGACCATGCTTGA
MIGA2O3	TGAAGGATAGGTGTGGTG	CATGAGAGAGAGAGAGAGA
MIGA224	CACGAGAGAGAGAGAGAGA	GGGTCTCAGAGGGAGGATTT
MIAC251-1	CCTTGGGTTCATTCGCTAAA	GGACGCCACACACACACAC
MIAC251-2	TGGCGCTACACACACACAC	CACACACACACACACACACG
LMMA8	CATGGAGTTGTGATACCTAC	CAGAGTTAGCCATATAGAGTG
MillHR04c	CGTTTTTGACCCTCTTGAGC	CCGCATACTTCCCTTCACAT
MillHR06	CGCCGAGCCTATAACCTCTA	ATCATGCCCTAAACGACGAC
MillHR07a	GCCACTCAGCTAAATAGCCTCT	TGCAGTCGGTAAAGTGATGG
MillHR11a	CAGTGAAACCACCAGGTCAA	TGGCCAGCTGATACCTTCTT
MillHR20a	CCTAACGCGCAAGAAACATA	ACCCACCTTCCCAATCTTTT
AJ635164	AAACAAAGAATGGAGCA	TGGACTGAATGTGGATAG
AY942826	TGTGAAATGGAAGGTTGAG	ACAGCAATCGTTGCATTC
AJ635178	GTATAAATCGCGTGCAT	AGTTTCCCTCCTTGTATCT
AJ635187	ATCCCCAGTAGCTTTGT	TGAGAGTTGGCAGTGTT
AY942817	TAACAGCTTTGCTTGCCTCC	TCCGCCGATAAACATCAGAC
AY942825	CGAGGAAGAGGAAGATTATGAC	CGAATACCATCCAGCAAAATAC
AJ635166	CTTGAAAGAGATTGAGATTG	AGAAGGCAGAAGGTTTAG
AJ635184	TGTCTACCATCAAGTTCG	GCTGTTGTTGCTTTACTG
AY942820	AGGTCTTTTATCTTCGGCCC	AAACGAAAAAGCAGCCCA
AB190349	AATTATCCTATCCCTCGTATC	AGAAACATGATGTGAACC
AY942828	CTCGCATTTCTCGCAGTC	TCCCTCCATTTAACCCTCC
AJ635189	ACGGTTTGAAGGTTTTAC	ATCCAAGTTTCCTACTCCT

2.4. PCR Amplification and SSR Fragment Analysis

PCR was completed with all 50 SSR primers pairs and 200 samples of variety used in this study. PCR was performed 25µL reaction volumes containing 12µL of 2x Green PCR master mix, 0.6 uM forward and reverse primers (approximately 25 ng of gDNA), and 50ng of gDNA as a template. Amplification was performed in a Thermal cycler (eppendorf Mastercycler gradient). The Mastercycler was programmed to pre-denaturation step of 94°C for 5 min followed by 35 cycles of denaturation 94°C for 30 sec, approximately annealing 55-60°C for 1 min (varied with Tm of different primers) and 72**°C** for 1 min followed by a final synthesis at 72 °C for 5 min. The reactions were then held at 4 °C. Amplifications were performed for twice and only reproducible products were considered for further data analysis.

2.5. Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

In order to explore genetic polymorphism, 3µL of denatured SSR-PCR mixture was resolved on 6% Polyacrylamide (19:1 acrylamide: bis-acrylamide) Gel Electrophoreses (PAGE) (for high resolution). The 50bp DNA ladder (Fermentas, USA) was used as a molecular size marker. The amplified bands were visualized by silver nitrate staining in an ethidium bromide solution as

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described [21]. The gel profile was photographed under UV light as digital images using a gel documentation and analysis system.

2.6. Band Recording DATA Analysis for DNA Fingerprinting

The binary data matrices obtained from SSR markers were processed at DN fingerprinting level. The presence of band will be scored as 1, whereas the absence will be scored as 0. The binary data matrix will be used for dendrogram construction. Cluster analysis was performed on the similarity coefficient matrix. The Jaccard similarity matrix was used for cluster analysis using Unweighted Pair Group Method of Arithmetic average (UPGMA)[27] into Numerical Taxonomy System of Multivariate Programs (NTSyspc) (version 2.10e) software package [28]. Exact size of DNA fragment was recorded for each variety and primer. The distinct bands are identified and labeled as DNA fingerprints.

2.6. Staistical Analysis

The amplified SSR bands resulting from the SSR-PCR were summarized as graphical representations using R-language (version 3.1.1, software version 3.5.1)[29].

3. Results

3.1. Genetic Amplification of Mango Cultivars Using SSR Markers

A total of 50 pairs of polymorphic SSR markers were selected to process two hundred leaf samples of DNA from each standard cultivar and candidate mango line 'Azeem Chaunsa' as templates (Table 1). The polymorphic SSR marker primers were selected to amplify and distinguish bands for screening. Out of 50 polymorphic SSR primers, 47 primers pairs amplified 82 SSR fragments from the candidate Azeem Chaunsa. Similarly 49 primers set amplified a total of 105 SSR fragments in Sufaid Chaunsa genome. The 41 primers amplified 62 polymorphic bands in Sindhri, and each primer pair amplified an average of 1.5 polymorphic fragments. The 41 primers amplified 70 polymorphic bands in S.B Chaunsa, and each primer pair amplified an average of 1.7 polymorphic fragments. A total of 319 DNA fragments were obtained across all genotypes using the 50 SSR primers. A set of 34 SSR primers showed amplification in all cultivars. Similarly, 40 common fragments were amplified in all cultivars (**Figures 1 and 2 and Table 2**)

Table 2. SSR-PCR amplification profile of 50 SSR mango markers on gDNA of four mango cultivars resulted different fragments obtained in this study. SSR loci that distinguish Azeem Chaunsa and standard mango cultivars.

SSR Marker ID	Sufaid Chaunsa	Sindhri	SB Chaunsa	Azeem Chaunsa
LMMA1F	290, 295	310	290, 295, 310	295
LMMA7F	260	205, 220, 260, 340	220, 340	220, 340
LMMA9F	205	0	200, 205	200
mMiCIRO14	210	205	0	**160**, 200, 210
mMiCIRO18	195, 240, 250, 350	380	250	250, 280
mMiCIRO32	190, 200	200	190, 200	190, 200
MiSHRS-1	180	175, 180	175, 240	210
MiSHRS-48	180, 190, 200	180	180, 210, 220	180, 200, 280
MIAC-4	90, 125	90, 125	90	90, 100, 125
MIAC-6	250	0	250	390
MGDSSR1	205	205	205	205
MGDSSR2	260, 270	260	150, 260	0
MGDSSR5	155, 295	155, 190, 300	160, 300	300
MGDSSR11	190, 240, 390	190, 240, 390	200, 240	190, 240
MGDSSR14	150, 200, 225, 250	150	150, 225	200

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MGDSSR34	150, 190	150, 190	150, 190	100, 150, 175, 180
MillHR21	140, 310, 400, 425	400	140, 400, 425	140, 310, 400, 425
mMiCIR005	210, 240, 250	210, 240	250	230
mMiCIR009	175, 240	175, 220, 240	220	220
mMiCIR013	160, 220	160, 220	160, 220	160, 220
mMiCIR016	250, 260, 280, 360	260	250, 260, 280	250
mMiCIR030	230, 245, 250	245, 250, 295	180, 245, 290, 295	180, 290
MiSHRS-37	200, 220	200	140, 245	140, 200
MIAC-11	145, 150	145, 150	145, 150	145
MITGI75	110, 150, 175	175	0	100, 110
MITGg62	450	175, 200	200	170, 200, 450
MICA231-1	300	600	195, 300, 620	320, 600
MICA235	120, 200, 400	0	400	200
MIGA2O3	155, 275	155	155	155, 275, 380
MIGA224	250, 300	250	300	250, 300
MIAC251-1	350, 600, 700	350, 600	350, 600	350, 600, 700
MIAC251-2	200	175, 200	175, 200	200
LMMA8	480	430	0	430
MillHR04c	160, 250	160	0	160
MillHR06	105	0	120	105
MillHR07a	160	0	160	160
MillHR11a	190, 220, 290	220	220	190, 220
MillHR20a	0	0	0	190
AJ635164	240, 380	240	240	240, 380
AY942826	225	0	0	225
AJ635178	240	0	0	0
AJ635187	240, 250	290	290	240
AY942817	200, 210, 250	200	190, 200	210, 250
AY942825	230, 260, 280	260	0	260
AJ635166	225, 250, 290	225	225	225, 290
AJ635184	160, 165, 190	175	165	165, 175
AY942820	200, 205, 250	205, 250	200, 250	205, 250
AB190349	130	130	130	0
AY942828	130, 135, 160	0	0	135
AJ635189	145, 155	145	145	145, 155

Out of fifty primers of polymorphic SSR markers, only 47 primers showed amplification in Azeem Chaunsa, 41 primers showed amplification in Sindhri and SB Chaunsa. The highest efficiency of primers observed in Sufaid Chaunsa (**Figure 1**).

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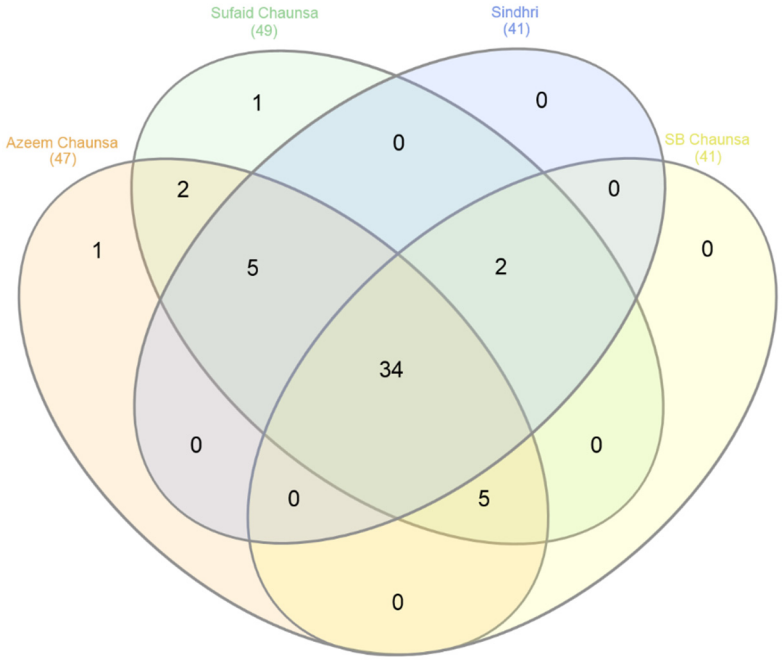

Figure 1. Venn Diagram of SSR marker primer pairs showing amplifiction in mango cultivars. SSR primers amplified different fragments in four cultivars: Sufaid Chunsa, Sindhri, S.B Chunsa and Azeem Chunsa. The degree of ovelap between mango cultivars was obserevd at primer level. The intersetcion of four cultivars showed 34 common SSR primers pairs.

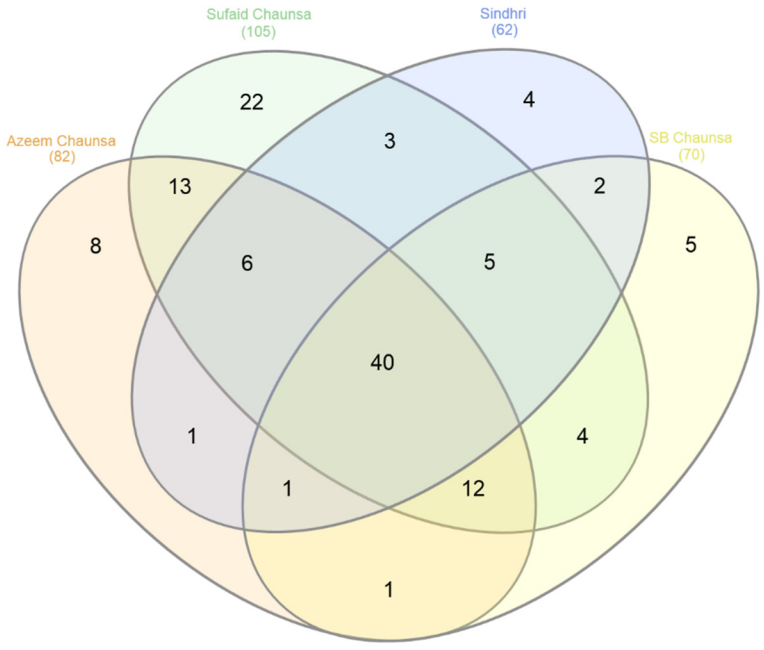

Figure 2. Venn Diagram showing common and exclusive fragments obtained from mango cultivars genomes. SSR primers amplified different fragments in four cultivars: Sufaid Chunsa, Sindhri, S.B

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Chunsa and Azeem Chunsa. The degree ovelap between mango cultivars was obserevd at fragment level. The intersetcion of four cultivars showed 40 common fragments amplified by SSR primer pairs.

Seven SSR marker showed highest amplification of fragments in all cultivars; MiSHRS-48, MGDSSR11, MGDSSR34, MillHR21, mMiCIR016, mMiCIR030, and MIAC251-1. Some SSR marker primers showed very low amplification of fragments: MillHR20a and AJ635178 (**Table 2**).

3.2. Distribution of Unique SSRs with Polymorphism

The 50 SSR primer pairs generated a total of 154 alleles with an average of 3.08 alleles per primer pair in all cultivars of Mango. Out of 154 alleles, 130 were found polymorphic alleles. The highest allele size range was observed in following SSR markers: mMiCIRO18, MGDSSR11, MillHR21, MITGg62, MICA231-1, MICA235, MIGA2O3, MIAC251-1, and AJ635164. The highest number of alleles was generated by SSR markers in mango cultivars: mMiCIRO18, MiSHRS-48, MGDSSR5, MGDSSR34, mMiCIR030, and MICA231-1. The highest number of polymorphic alleles was mMiCIRO18, MiSHRS-48, MGDSSR5, mMiCIR030, and MICA231-1 (**Table 3**). The highest rate of polymorphism generated by following SSR markers: mMiCIRO18, MGDSSR5, mMiCIR030, and MICA231-1 (**Table 3**).

Table 3. Allele distribution, polymorphism and diversity in four mango cultivars.

SSR Primer Pair ID	Tm °C	Allele Size (bp)	No. of Loci	No. of Polymorphic loci	Polymorphic loci %
LMMA1F	59	290-310	3	3	100
LMMA7F	55	205-340	4	4	100
LMMA9F	56	200-205	2	2	100
mMiCIRO14	57	160-210	4	4	100
mMiCIRO18	59	195-380	6	6	100
mMiCIRO32	57	190-200	2	1	50
MiSHRS-1	65	175-240	4	4	100
MiSHRS-48	57	180-280	6	5	83.33
MIAC-4	59	90-125	3	2	66.66
MIAC-6	65	250-390	2	2	100
MGDSSR1	62	205	1	0	-
MGDSSR2	65	150-270	3	3	100
MGDSSR5	65	155-300	5	5	100
MGDSSR11	65	190-390	4	3	75
MGDSSR14	65	150-250	4	4	100
MGDSSR34	65	100-190	5	4	80
MillHR21	64	140-425	4	4	100
mMiCIR005	58	210-250	4	4	100
mMiCIR009	57	175-240	3	3	100
mMiCIR013	60	160-220	2	0	-
mMiCIR016	58	250-360	4	4	100
mMiCIR030	55	180-295	6	6	100
MiSHRS-37	65	140-245	4	4	100
MIAC-11	61	145-150	2	1	50
MITGI75	65	100-175	4	4	100
MITGg62	59	170-450	4	4	100
MICA231-1	65	195-600	5	5	100
MICA235	65	120-400	3	3	100
MIGA2O3	60	155-380	3	2	66.66
MIGA224	63	250-300	2	2	100
MIAC251-1	64	350-700	3	1	33.33

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MIAC251-2	65	175-200	2	1	50
LMMA8	60	430-480	2	2	100
MillHR04c	65	160-250	2	2	100
MillHR06	65	105-120	2	2	100
MillHR07a	65	160	1	0	0
MillHR11a	65	190-290	3	2	66.66
MillHR20a	64	190	1	0	0
AJ635164	56	240-380	2	1	50
AY942826	60	225	1	0	0
AJ635178	57	240	1	0	0
AJ635187	61	240-290	3	3	100
AY942817	65	190-250	4	4	100
AY942825	62	230-280	3	2	66.66
AJ635166	56	225-290	3	2	66.66
AJ635184	59	160-190	4	4	100
AY942820	64	200-250	3	2	66.66
AB190349	57	130	1	0	0
AY942828	65	130-160	3	3	100
AJ635189	58	145-155	2	1	50

3.3. SSR Fingerprinting/ Allelic Diversity

Four mango cultivars were DNA fingerprinted. The SSR profiling of highly diverse candidate line Azeem Chaunsa cultivar exhibited polymorphism using 50 SSR markers. Out of 50 SSR, 47 SSR primer pairs yielded strong amplification in candidate cultivar. The allele size varied from 90 bp in MIAC-4 to 700 bp in MIAC251-1 in Azeem Chaunsa cultivar. The number of alleles per marker varied from 1 (LMMA1F) to 4 (MGDSSR34, MillHR21).

In total, 82 SSR alleles were amplified in the candidate line Azeem Chaunsa using 50 SSR markers. Out of 82 SSR alleles, 60 SSR alleles were detected as polymorphic. The SSR alleles of 160 bp, 200 bp (marker name mMiCIRO14), 280 bp (mMiCIRO18), 210 bp (MiSHRS-1), 280 bp (MiSHRS-48), 100 bp (MIAC-4), 390 bp (MIAC-6), 100 bp, 175 bp, 180 bp (MGDSSR34), 230 bp (mMiCIR005), 100 bp (MITGI75), 170 bp of (MITGg62), 320 bp (MICA231-1), 380 bp (MIGA2O3), 190 bp (MillHR20a) were amplified only in Azeem Chaunsa genome. The analysis revealed a total of 60 polymorphic alleles ranging from 1 to 3 per locus, with an average of 1.2 alleles per locus in candidate line (**Table 4**). However, there are four SSRs i.e., mMiCIRO14, MGDSSR34, MillHR21 and MITGg62 which yielded 3 alleles per locus.

Table 4. Allele distribution and polymorphism was estimated in Azeem Chaunsa cultivar.

SSR Loci ID	Nature	Polymorphic type	Polymorphic alleles (N.)	Allele size (bp)
LMMA1F	polymorphic	co-dominate	1	295
LMMA7F	polymorphic	co-dominate	2	220, 340
LMMA9F	polymorphic	co-dominate	1	200
mMiCIRO14	polymorphic	co-dominate	3	160, 200, 210
mMiCIRO18	polymorphic	co-dominate	2	250, 280
mMiCIRO32	polymorphic	co-dominate	1	190, 200
MiSHRS-1	polymorphic	co-dominate	1	210
MiSHRS-48	polymorphic	co-dominate	2	180, 200, 280
MIAC-4	polymorphic	co-dominate	2	90, 100, 125
MIAC-6	polymorphic	co-dominate	1	390

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MGDSSR1	monomorphic	dominant	0	205
MGDSSR2	polymorphic	co-dominate	0	0
MGDSSR5	polymorphic	co-dominate	1	300
MGDSSR11	polymorphic	co-dominate	1	190, 240
MGDSSR14	polymorphic	co-dominate	1	200
MGDSSR34	polymorphic	co-dominate	3	100, 150, 175, 180
MillHR21	polymorphic	co-dominate	3	140, 310, 400, 425
mMiCIR005	polymorphic	co-dominate	1	230
mMiCIR009	polymorphic	co-dominate	1	220
mMiCIR013	polymorphic	co-dominate	0	160, 220
mMiCIR016	polymorphic	co-dominate	1	250
mMiCIR030	polymorphic	co-dominate	2	180, 290
MiSHRS-37	polymorphic	co-dominate	2	140, 200
MIAC-11	polymorphic	co-dominate	0	145
MITGI75	polymorphic	co-dominate	2	100, 110
MITGg62	polymorphic	co-dominate	3	170, 200, 450
MICA231-1	polymorphic	co-dominate	2	320, 600
MICA235	polymorphic	co-dominate	1	200
MIGA2O3	polymorphic	co-dominate	2	155, 275, 380
MIGA224	polymorphic	co-dominate	2	250, 300
MIAC251-1	polymorphic	co-dominate	1	350, 600, 700
MIAC251-2	polymorphic	co-dominate	0	200
LMMA8	polymorphic	co-dominate	1	430
MillHR04c	polymorphic	co-dominate	1	160
MillHR06	polymorphic	co-dominate	1	105
MillHR07a	monomorphic	dominant	0	160
MillHR11a	polymorphic	co-dominate	1	190, 220
MillHR20a	monomorphic	dominant	0	190
AJ635164	polymorphic	co-dominate	1	240, 380
AY942826	monomorphic	dominant	0	225
AJ635178	monomorphic	dominant	0	0
AJ635187	polymorphic	co-dominate	1	240
AY942817	polymorphic	co-dominate	2	210, 250
AY942825	polymorphic	co-dominate	1	260
AJ635166	polymorphic	co-dominate	1	225, 290
AJ635184	polymorphic	co-dominate	2	165, 175
AY942820	polymorphic	co-dominate	1	205, 250
AB190349	monomorphic	dominant	0	-
AY942828	polymorphic	co-dominate	1	135
AJ635189	polymorphic	co-dominate	1	145, 155

3.5. DNA Fingerprinting Analysis

The genetic relation at DNA fingerprint level among the standard cultivars and candidate line was evaluated using cluster analysis. The cultivar identification diagram (CID) was constructed using UPGMA algorithm for the evaluation of genetic diversity and relatedness among the mango cultivars. CID presenting association among standard cultivars (Sufaid Chaunsa, Sindhri and S.B Chaunsa) based on the phylogenetic relationship using coefficients by NTSYS cluster analysis (Figure 3). Dice similarity coefficients were calculated for the 50 SSR markers, and a UPGMA tree was generated (Figure 3). Cluster I consists of Azeem Chaunsa and further divided into two cultivars, S.B Chaunsa and Sindhri. Cluster II consists of Sufaid Chaunsa. Cluster III consisted of Chenab-Gold. X-

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axis represents similarity coefficient between genotypes with ranged from 0.49-0.67. CID results concluded that candidate cultivar 'Azeem Chaunsa' varied significantly from the standard cultivar Sufaid Chaunsa (46.7% dissimilarity), Sindhri (46.2% dissimilarity) and SB Chaunsa (45% dissimilarity)

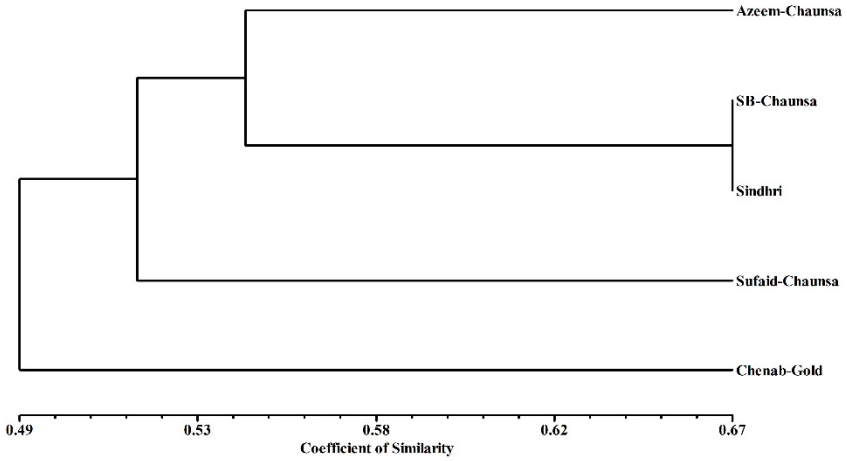

Figure 3. Cultivar identification diagram (CID) based on hierarchical NTSYS cluster estimating Jaccard's similarity coefficient. The UPGMA based CID shows the clustering and association of mango cultivars based on SSR marker data.

4. Discussion

The standard cultivars included in the present study probably represent a major component of the mango gene pool in Pakistan. Further, SSR markers have been broadly used in mango genetic research to differentiate cultivars, hybrids and to evaluate new varieties [13,14]. The improvement in genetic and agronomic traits for high yield potential in fruit plants is highly based on the proper assessment of diversity analysis. Systematic mango breeding is laborious, time-consuming and is a long-term endeavor (up to 25 years) due to a highly heterozygous genome as well as long juvenility. Promising selection, introduction, evaluation of cross-breeding progenies and mutational breeding has been widely used to develop mango cultivars, varieties and hybrids [23,30,31]. In current study, a mango candidate line was developed and further molecular diversity was analyzed with three standard cultivars using SSR markers. Highly unique, diverase cultivars of mango have been grown in Punjab and Sindh provinces of Pakistan as they have a long history of breeding. In mango, variety identification has been greatly challenging. The SSR markers are very sensitive to evaluate hybrid mango lines to identify genetic contamination. Several microsatellite as molecular markers such as SSR have been developed for the Mango [18,24,26]. These markers have shown to be reliable, consistent and reasonably discriminative for use by several laboratories as a mango genotyping tool. In this study, 50 pairs of SSR primers were used for PCR amplifications of different bands in all four mango cultivars (**Tables 1 and 2**). Among these primers, 45 pair of primers showed amplification in the candidate line 'Azeem Chaunsa' as shown (**Figure 1** and **Table 2**). The highest polymorphic ratio of the SSR primers are associated with the hyper variable nature of the SSR markers. The maximum number of polymorphic bands was obtained by the mMiCIRO18, MiSHRS-48, MGDSSR5, mMiCIR030, mMiCIR030, and MICA231-1 SSR primers (**Table 3**). MillHR21 and mMiCIR030 primers recorded the highest (twelve) amplified bands. While the lowest (one) numbers of amplified bands was recorded in MillHR20a and AJ635178 (**Table 2**). The results are in agreement with previous reports based on SSR markers developed for *Mangifera indica* to evaluate mango varieties in India [18,32,33], China [14,34], Indonesia [35], Pakistan [3,36], Maxico [37] and Japan [13].

The polymorphism ratio of amplified alleles was observed very high, and several unique alleles were identified in the candidate line which provides important basis for subsequent use these primers. The 45 primer pairs generated clear single-locus polymorphic bands and 5 primers pairs

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yielded monomorphic bands in the candidate line. The 50 SSR primer pairs generated a total of 319 fragments in 4 mango cultivars with an average of 6.38 fragments per primer. Notably, primers MGDSSR1, mMiCIR013, MillHR07a, MillHR20a, AY942826, AJ635178 and AB190349 did not present any polymorphic bands (**Tables 2 and 3**). These results are in agreement with previous studies on mango [13,14].

Generally for UPGMA based cultivar identification and the construction of dendrogram, more than 10 markers were used [38]. Therefore, these highly polymorphic SSR primer pairs can be applied as core primer pairs for variety identification. In this study, DNA fingerprints of the 4 mango cultivars were constructed according to the original data matrix of amplification results (**Figure 3**). The number of bands produced across 4 mango varieties by different SSR primers is consistent with published reports on microsatellite frequency in the mango genome [13,14].

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