

Full Length Article

Microsatellite (SSR) Markers Application in the Characterization of Genetic Diversity in *Capsicum annuum* Germplasm

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Abstract

This study focused on the genetic diversity of *Capsicum annuum* (commonly known as chili or bell pepper) using 40 Simple Sequence Repeat (SSR) markers across 133 genotypes. Out of 40 SSR markers, 29 were polymorphic, indicating considerable genetic variation within the genotypes. The polymorphic information content (PIC) values ranged from 0.098 to 0.992, with an average of 0.708, reflecting a broad spectrum of genetic information. Notably, 22 markers had PIC values greater than 0.5, demonstrating strong discriminatory power. Among SSRs, the marker AVRDC-17 exhibited the highest PIC value at 0.992, followed by AVRDC 174 (0.990), Agi96 (0.967), and HPMS 1-5 (0.961). The expected heterozygosity (He) of the genotypes varied from 0.038 to 0.766, with an average of 0.428. The marker HPMS 1-214 showed the highest expected heritability value of 0.766, a high level of genetic variability in certain genotypes. Cluster analysis grouped the genotypes into seven distinct genetic clusters, reinforcing the presence of considerable genetic differentiation among the genotypes. Genetic similarity coefficients ranged from 0.11 to 0.91, reflecting the diverse genetic relationships within the population. The highest similarity was observed between genotypes 36569 and 36627, with a coefficient of 0.91, indicating a close genetic relationship. These findings confirm the usefulness of SSR markers in assessing genetic diversity, offering valuable information for breeding programs aimed at developing improved *Capsicum annuum* cultivars with enhanced yield and adaptability.

Keywords: Alleles; *Capsicum*; Genetic distance; Heterozygosity; Microsatellites; Polymorphic information content; PCR

Introduction

The species *Capsicum annuum* (commonly known as chili or bell pepper; local names: mirch and shimla mirch) belongs to the Solanaceae family, specifically in the tribe Solaneae and subtribe Capsicinae (Barboza *et al.* 2022). The nomenclature of *Capsicum* varieties varies based on type and region, with common names including red pepper, chili, bell pepper green, or simply pepper (Po *et al.* 2018).

Capsicum annuum is a key species in the *Capsicum* genus and is believed to have originated in South and Central America (Khan *et al.* 2020). Mexico is recognized as a primary center for the domestication and genetic diversity of *Capsicum*, holding cultivated varieties such as *Capsicum annuum* L. var. *annuum* and wild types like *Capsicum annuum* L. var. *glabriusculum* (Khouri *et al.* 2020). Its genus comprises of 25 wild species and 5 domesticated species, including *Capsicum annuum*,

Capsicum baccatum, *Capsicum pubescens*, *Capsicum chinense*, and *Capsicum frutescens*, with over 200 recognized varieties (Barchenger *et al.* 2019). Among these domesticated species, *Capsicum annuum* is the most cultivated species (Liu *et al.* 2024). Most species have a chromosome number of $2n = 2x = 24$ (Molla *et al.* 2022), although some wild species exhibit $2n = 2x = 32$ (Khan *et al.* 2020). All species are diploid.

For optimal growth, *Capsicum* plants thrive in temperatures between 20°C and 30°C in the daytime and prefer well-drained loamy soils with a pH between 5.5–6.8, which are enriched with lime (Khan *et al.* 2025).

Capsicum is one of the versatile plants grown as a vegetable and spice crop. It has become an essential ingredient in cuisines worldwide, valued for its pungency, color, and unique flavor (Khan *et al.* 2019). Its fruits are rich in oils, volatile and fatty (The word acid is missing with fatty). It must be fatty acid, and vitamins such as A, C, and E, as well as

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minerals including molybdenum (Mo), manganese (Mn), and potassium (K), along with folate and thiamine (Devi *et al.* 2021). The carotenoids in peppers serve as natural colorants, while capsaicinoids are widely utilized in food, medicine, and pharmaceuticals (Hernandez-Perez *et al.* 2020).

Furthermore, genetic resources are important for advancing crop improvement, with morphological markers utilized for variety identification and genetic diversity studies (Salgotra *et al.* 2023). However, the complex taxonomy of *Capsicum* can complicate genotype classification using only morpho-agronomic data (Mabuza *et al.* 2022). Molecular markers, *e.g.*, random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), restriction fragment length polymorphism (RFLPs) and simple sequence repeat (SSRs) markers offer valuable insights into genetic diversity and are instrumental for breeding and germplasm management (Kumar *et al.* 2024). The SSRs are particularly effective due to their wide distribution and high polymorphism within the *Capsicum* genome, providing precise discrimination of genetic relationships (Mimura *et al.* 2012; Negi *et al.* 2022). Despite the challenges and costs of genetic evaluation, successful characterization supports the development of improved cultivars and sustains crop diversity, particularly under diverse environmental conditions (Ceccarelli and Grando 2002). The improvement and application of genome-wide molecular markers in *Capsicum* provide a clear understanding of genetic relationships and molecular profiling of germplasm.

The objective of the study was to assess molecular diversity in *Capsicum* genotypes through the use of SSR (microsatellite) markers. This involved identifying genetic variations among the genotypes to select those with desirable traits. The ultimate goal was to identify promising genotypes for further breeding and improvement programs.

Materials and Methods

Experimental details

Experimental material: The study was conducted in the Plant Genomics Lab at the National Institute of Genomics and Advanced Biotechnology (NIGAB), located within the National Agricultural Research Centre (NARC) in Islamabad, Pakistan. A total of 133 *Capsicum* genotypes were obtained from the Gene Bank of the Bio-Resources and Conservation Institute (BCI) at NARC. The genotype numbers and their origins are presented in Fig. 1. Seed germination was initially conducted in the greenhouse at the BCI Farm in Islamabad. Once the seedlings reached an appropriate growth stage, they were transplanted into the field with a spacing arrangement of 45 cm and 75 cm between rows and plants. Throughout the cultivation period, standard agronomic practices, including fertilization and crop management techniques, were implemented to ensure optimal plant growth and productivity.



Fig. 1: Graphical representation of the genotypes

Plant material collection: Young leaves of *Capsicum* genotypes were collected from the field of NARC and were kept in a polystyrene (thermopole) box with an ice plate to be maintained at cold temperatures. The genotypes were labeled on plant samples. They were kept at -80°C until deoxyribonucleic acid (DNA) extraction was performed.

Genomic DNA extraction and microsatellite amplification

A cetyltrimethylammonium bromide (CTAB) method (Dolye and Dolye 1990) was utilized for the extraction of total genomic DNA. Leaf tissues of approximately 100 mg were homogenized in liquid nitrogen before adding the buffer. The genomic DNA quantity and quality were checked by a NanoDrop spectrophotometer. Further quantity and quality of DNA were confirmed by electrophoresis of a sample with a known quantity of standard.

Nanodrop spectrophotometer: The DNA concentration was measured on a NanoDrop, ND-1000 spectrophotometer. The 260 nm peak absorbance was exhibited by nucleic acid, whereas the 280 nm absorbance was recorded for proteins. Readings were recorded at both wavelengths, and the purity of the DNA was quantified in terms of the OD260/OD280 ratio. A reading between 1.8 to 2.0 indicated minimal protein contamination, signifying high DNA purity. The DNA quantity in a pure sample was determined based on the principle that the value of 1.0 absorbance at 260 nm is equivalent to 50 µg of double-stranded DNA per mm of solution (Li *et al.* 2014). The concentration of DNA (µg/mL) was then calculated using the formula: OD260 × 50.

Selection of primers and polymerase chain reaction (PCR) optimization

A subset of SSR marker pairs was selected from each linkage group based on the established microsatellite framework for *Capsicum annuum* fingerprinting (Table 1).

Table 1: List of SSR markers utilized in the study

Primer ID	Forward primer	Reverse primer	Reference	Polymorphic/non-polymorphic
AGi96	CTGCCCTCCTCAACCCTAA	CGGTCACTGGACTCTGATGTT	Nugroho <i>et al.</i> (2019)	Polymorphic
AVRDC PP 03	CTCGATGACTTGATCGTGA	CTTGCACTGTGAGGTCACTG	Dhaliwal <i>et al.</i> (2014)	Polymorphic
HpmSE31	CCCTAAATCAACCCCAATTCAA	CCCCATTACCTGACTGCAAAA	Verma <i>et al.</i> (2024)	Polymorphic
CAMS-162	GGACCGTTTCAGGAGGTTACA	GCCATCATTCAAAACCGAAT	Dhaliwal <i>et al.</i> (2013)	Polymorphic
HpmsCaSIG-19	CATGAATTTCTGCTTTGAAGGTCCTC	AAGGGTGTATCGTACGCAGCCTTA	Dhaliwal <i>et al.</i> (2013); Toledo-Aguilar <i>et al.</i> (2016)	Polymorphic
CA5t 6439	GACAGTCTTTCAGAACTAGAGAGAG	TGGAGCAAACACAGCAGAAC	Ozturk <i>et al.</i> (2019)	Polymorphic
Hpms1-5	CCAAACGAACCGATGAACACTC	GACAATGTTGAAAAAGGTGGAAGAC	Kwon <i>et al.</i> (2005)	Polymorphic
Hpms 1-1	TCAACCCAATATTAAGGTCACCTCC	TCAACCCAATATTAAGGTCACCTCC	Kwon <i>et al.</i> (2005); Nugroho <i>et al.</i> (2019)	Polymorphic
Bd12 Hpms1-139	CCAACAGTAGGACCCGAAAATCC	ATGAAGGCTACTGCTGCGATCC	López-Candelo <i>et al.</i> (2022)	Polymorphic
CAMS-234	TATAGCCCATGGGTGCCTTT	AAAACCCAATATTAACCATATGCAA	Indrabi <i>et al.</i> (2024)	Polymorphic
CAMS-311	GGTGCCTAGAGATGGAGAG	TTTGAGTGTTCGGGACTGGT	Dhaliwal <i>et al.</i> (2013)	Polymorphic
CAMS-838	CCAGGATGGTGTAAAGGGTTT	GTCGCATCAATGAGCATAGG	Minamiyama <i>et al.</i> (2006)	Polymorphic
CAMS-647	CGGATTCGGTTGAGTCGATA	GTGCTTTGGTTCGGTCTTTC	Haque <i>et al.</i> (2021)	Polymorphic
CAMS-644	CGCATGAAGCAAATGTACCA	ACCTGCAGTTTGTGTGGA	Minamiyama <i>et al.</i> (2006)	Polymorphic
BM067271	GTATGCTGCAACCATCGTTG	ATTGGTTTGGGAGACACAGC	Minamiyama <i>et al.</i> (2006)	Polymorphic
Bd70-CMS 0005	CATGACCACCATGAGGATA	GATAGCCACGAGCATAGTATT	Guzman <i>et al.</i> (2020)	Polymorphic
CAMS-90	(ca)3a(ac)15		Leite <i>et al.</i> (2016)	Polymorphic
CAMS-153	TGCACAAATATGAATCCCAAGA	AAGTCAGCAAACACATCTGACAA	Minamiyama <i>et al.</i> (2006)	Polymorphic
Hpms2-23	CCCTCGGCTCAGGATAAAATACC	CCCCAGACTCCCACTTTGTG	Lee <i>et al.</i> (2004)	Polymorphic
Ng8 (CA516044)	ATCTTCTTCTCATTTCTCCCTTC	TGCTCAGCATTAACGACGTC	López-Candelo <i>et al.</i> (2022)	Polymorphic
CAS15275	CTCTGCCCTCCTCAACCC	AAAATATGGTCGGAGATCCG	Dhaliwal <i>et al.</i> (2014)	Polymorphic
AVRDCPP 17	CTACTACCGCTCCTGCTCCT	AGCTTCTGCTTTTGGTTTCGT	Dhaliwal <i>et al.</i> (2014)	Polymorphic
AVRDCPP 174	TCGTTGTTGGGTGGTACTTG	GGAAGATCTCAAATGGGTGCG	Dhaliwal <i>et al.</i> (2014)	Polymorphic
AVRDCPP 67	TATTCCTTCTCACCCCTCC	GAAAGAGGGCGCTAAGTGGAC	Terefe <i>et al.</i> (2022)	Polymorphic
Ng18 (CA517699)	ACGCCAAGAAATCATCTCC	CCATTGTGAAGAAATGGG	López-Candelo <i>et al.</i> (2022)	Polymorphic
Hpms2-13	TCACCTCATAAGGGCTTATCAATC	TCCTTAACCTTACGAAACCTTGG	Toledo-Aguilar <i>et al.</i> (2016)	Polymorphic
Hpms1-148	GGCGGAGAAGAACTAGACGATTAGC	CCACCCAATCCACATAGACG	Kwon <i>et al.</i> (2005)	Polymorphic
Hpms1-155	ACGAGGCCAAGCTGTTATGTC	TTGTCCCGACTCTCCATTGACC	Hazarika <i>et al.</i> (2023)	Polymorphic
Bd22-Hpms1-214	TGCGAGTACCCGAGTTCTTTCTAG	GGCAGTCTGGGACAACCTCG	López-Candelo <i>et al.</i> (2022)	Polymorphic
AVRDC PP 18	GCTAGGCTTGATCCTTCACC	CGCTTGAATCATGCTCACT	Dhaliwal <i>et al.</i> (2014)	Monomorphic
AVRDC PP 24	AAAGCATGAAATCACCTCC	CGGCAAGAAGATGAAAGTCA	Dhaliwal <i>et al.</i> (2014)	Monomorphic
CAMS-864	CTGTTGTGAAGAAGAGGACA	GCTTCTTTTCAACCTCCTCCT	Haque <i>et al.</i> (2021)	Monomorphic
CAMS-072	CCCGCGAAATCAAGGTAAT	AAAGCTATTGCTACTGGGTTCG	Dhaliwal <i>et al.</i> (2014)	Monomorphic
AVRDC PP 205	AACCCCTTCAAACCTGTTGC	GGGGTTCGAAGTAGATGAA	Dhaliwal <i>et al.</i> (2013)	Monomorphic
AGi42	GGGAAGAGAAATTTGTGAAAGCA	ATGCCAACAATGGCATCCTA	Usman <i>et al.</i> (2018)	Monomorphic
CAMS-398	ATGGTCCATGGTCAGCAGAT	GGGCAGAACAGTGGATGATT	Ibiza <i>et al.</i> (2012)	Monomorphic
CAMS-605	CCAGTCTACCCAGCAGACA	CATATGCTGCTCCTGATTTC	Haque <i>et al.</i> (2021)	Monomorphic
Bd17 Hpms1-165	GGCTATTTCCGACAAACCTCAG	CCATTGGTGTTTTCACTGTTGTG	Lee <i>et al.</i> (2004)	Monomorphic
CAMS-806	GCTAGGCTTGATCCTTCACC	CGCTTGAATCATGCTCACT	Sharmin <i>et al.</i> (2018)	Monomorphic

The selection criteria for the microsatellites included robustness, a preference for single-locus markers, and evidence of polymorphism in prior studies. The conditions for PCR were optimized to ensure robust amplification.

The reaction mixture (20 μ L) for PCR consisted of 1 μ L of total DNA, 2 μ L of 10 $\times$ buffer, 0.4 μ L of mixed dNTPs, 1 μ L each of forward and reverse primers, 1.6 μ L of $MgCl_2$, 0.2 μ L of Taq polymerase, and double-distilled water (12.8 μ L). Amplification was performed in a PCR cycler programmed with an initial hot start at 95°C for five min, followed by 1-min denaturation at 94°C for thirty-five cycles, annealing for one min at 55°C, and elongation for two min at 72°C. A final extension was carried out at 72°C for 10 min, with the reactions stopped at 4°C until electrophoresis.

Gel scoring

The (15 μ L) PCR products were resolved on agarose gels (1.5–3%), and staining was done with ethidium bromide in

1 $\times$ TBE buffer (1 mM EDTA and 10 mM Tris-Borate) for the separation of amplified bands. A 100 base pair ladder was used to estimate product sizes. An ultraviolet (UV) transilluminator was used for the visualization of bands. Bands were scored for the presence or absence.

Statistical analysis

DNA bands were recorded using a binary coding system, where the presence of a band was assigned a value of 1 and the absence a value of 0, employing PAST software version 4.01. Gene diversity was calculated following the methodology established by Nei and Li (1979). The PIC value for each marker was computed based on Nei's statistics (Nei 1973) in Excel, using the formula:

$$PIC = 1 - \sum (P_{ij}^2)$$

Where, P_{ij} represents the frequency of the j-th allele at the i-th primer, with the summation extending over 'n' patterns.

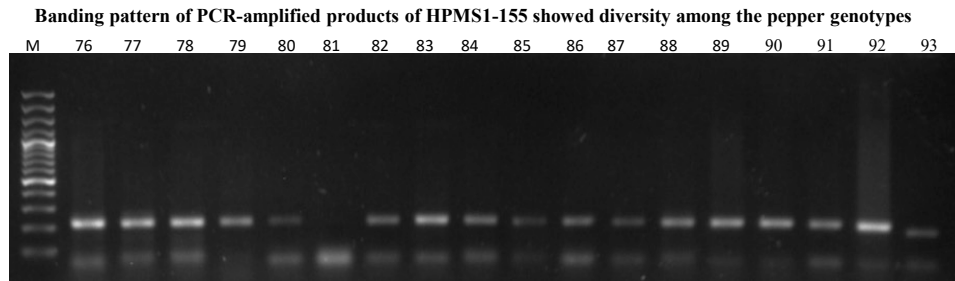


Fig. 2: Banding pattern of PCR-amplified products of HPMS1-155 showed diversity among the pepper genotypes. Lanes M 100 bp ladder, 76: 36552, 77: 36111, 78: 30878, 79: 36118, 80: 36641, 81: 36110, 82: 36545, 83: 36640, 84: 33832, 85: 36648, 86: 36650, 87: 36659, 88: 36554, 89: 36631, 90: 36119, 91: 36626, 92: 36629, 93: 36634

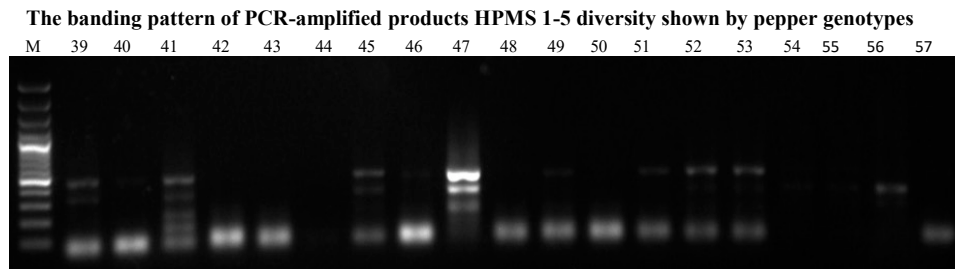


Fig. 3: The banding pattern of PCR-amplified products HPMS 1-5 diversity shown by pepper genotypes. Lanes M 100 bp ladder, 39: 36598, 40: 36573, 41: 36113, 42: 36633, 43: 36613, 44: 36609, 45: 30882, 46: 47: 36116, 48: 36638, 48: 36621, 49: 36625, 50: 36610, 51: 36611, 52: 36608, 53: 36603, 54: 36607, 55: 36606, 56: 36604, 57: 36601

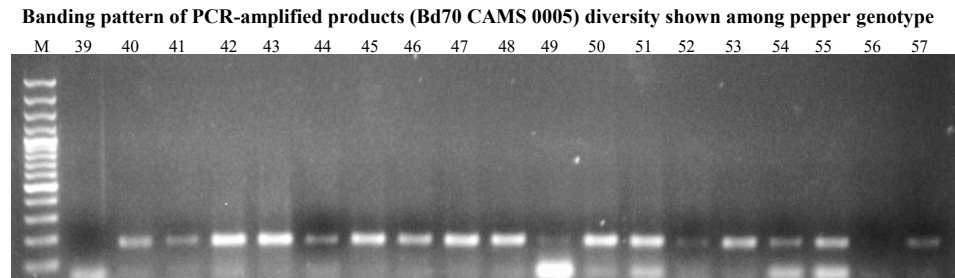


Fig. 4: Banding pattern of PCR-amplified products (Bd70 CAMS 0005) diversity shown among pepper genotypes. Lanes M 100 bp ladder, 39: 36598, 40: 36573, 41: 36113, 42: 36633, 43: 36613, 44: 36609, 45: 30882, 46: 47: 36116, 48: 36638, 48: 36621, 49: 36625, 50: 36610, 51: 36611, 52: 36608, 53: 36603, 54: 36607, 55: 36606, 56: 36604, 57: 36601

The classification for polymorphism was as follows: PIC value less than 0.25 indicates low polymorphism, whereas $0.25 \leq$ and < 0.5 PIC signifies moderate polymorphism, and ≥ 0.5 PIC represents high polymorphism (Serrote *et al.* 2022). Heterozygosity (He) was calculated using the formula:

$$H = 1 - \sum(p_i^2).$$

Results

Genetic diversity assessment utilizing SSR markers

A total of 40 SSR markers were initially screened, 29 exhibited polymorphism and were thus selected for subsequent genetic diversity analysis (Table 1). The remaining 11 markers, which were either monomorphic or failed to produce reliable amplification, were excluded from

further analysis. The 29 polymorphic SSR markers were applied to 133 *Capsicum annuum* genotypes to evaluate their effectiveness in amplifying distinct DNA fragments. These markers consistently generated clear and scorable banding patterns, facilitating the assessment of genetic variability among the genotypes. Representative amplification profiles obtained using markers HPMS1-155, HPMS1-5, and Bd70 CAMS0005 (Fig. 2-4, respectively), highlighting their polymorphic potential.

In this study, the 29 SSR markers revealed substantial variability, with a total of 2,504 bands identified across all genotypes, averaging 86.34 bands per marker. A total of 72 alleles were amplified, with an average of 2.48 alleles/locus. The maximum number of alleles observed was five, recorded for the primer HPMS 1-214, followed by four alleles for primers HPMS 1-5, CAMS 644, and HPMS2-23.

Table 2: Number of alleles per locus, heterozygosity and polymorphic information content values

Sr. No.	Primer's ID	Allele size	Na	He	PIC
1	Agi 96	200-250	2	0.349	0.967
2	AVRDC PP3	170-190	2	0.486	0.888
3	HPMSE31	230-250	2	0.491	0.664
4	CAMS 162	190-200	2	0.485	0.595
5	Hpms CASIG-19	200-217	2	0.359	0.368
6	CAST 6439	190-210	3	0.45	0.791
7	HPMS 1-5	100-418	4	0.645	0.961
8	HPMS 1-1	258-287	2	0.480	0.880
9	HPMS 1-139	190-240	3	0.361	0.720
10	CAMS 234	180-190	2	0.488	0.823
11	CAMS 311	200-234	2	0.499	0.920
12	CAMS 838	170-180	2	0.436	0.758
13	CAMS 647	220-245	2	0.363	0.782
14	CAMS 644	190-210	4	0.638	0.700
15	BM067271	290-300	2	0.433	0.612
16	CAMS 0005 Bd70	149-194	3	0.548	0.098
17	CAMS 90	210-245	3	0.362	0.733
18	CAMS 153	225-265	2	0.467	0.537
19	HPMS 2-23	200-265	4	0.484	0.660
20	NG8 (CAS516044)	182-250	2	0.316	0.424
21	CAS15275	101-105	2	0.274	0.389
22	AVRDC PP 17	190-200	2	0.260	0.992
23	AVRDC pp 174	190-210	2	0.493	0.990
24	AVRDC 67	200-210	2	0.4182	0.927
25	NG18(CAS 17699)	160-179	2	0.288	0.742
26	HPMS 2-13	219-259	2	0.301	0.833
27	HPMS-148	183-207	3	0.435	0.916
28	HPMS1-155	200-210	2	0.038	0.423
29	HPMS 1-214	100-300	5	0.766	0.456
Average			2.482	0.428	0.708

Additionally, five primers exhibited three alleles each and 20 primers displayed two alleles per locus (Table 2).

In our study, PIC values ranged from 0.098 to 0.992, with an average of 0.708 (Table 2). The highest PIC values were recorded for AVRDC 17 (0.992), AVRDC 174 (0.990), Agi96 (0.967), HPMS 1-5 (0.961), AVRDC 67 (0.927), CAMS 311 (0.920), and HPMS-148 (0.916). Other markers with relatively high PIC values included AVRDC PP3 (0.888), HPMS 1-1 (0.880), HPMS 2-13 (0.833), CAMS 234 (0.823), CAMS 647 (0.782), CAMS 838 (0.758), NG18 (CAS 17699) (0.742), CAM 90 (0.733), and CAM644 (0.700). Six SSR markers displayed PIC values below 0.5, including CAS15275, Hpms CASIG-19, HPMS 1-241, HPMS 1-55, and NG8 (CAS516044), while CAMS-555 Bd70 exhibited the lowest 0.098 PIC value.

In the present study, the expected heterozygosity (He) ranged from 0.038 to 0.766, with a mean value of 0.428. Among the markers, HPMS 1-214 showed the highest level of genetic diversity with He of 0.766, followed by HPMS 1-5 (He = 0.645) and CAMS 644 (He = 0.638). In contrast, the marker HPMS 1-155 had the lowest (0.038) He, reflecting minimal genetic diversity at the locus.

The analysis of SSR markers in our study identified a high level of polymorphism, with 72.5% of the markers displaying variability. This indicates a considerable degree of genetic variability among the pepper genotypes investigated.

Genetic distance matrix

The analysis of genetic distance among the pepper genotypes revealed substantial polymorphism at the DNA level. The distance coefficients, calculated from the data of twenty-nine SSR markers, varied widely from 0.00 to 1.00. The maximum (0.91) genetic distance was detected between genotypes 36569 and 36627. Other pairs with high genetic identity include 36616 vs. 36632 (0.89), and 36616 vs. 36628 and 36628 vs. 36632 (0.84). Conversely, the lowest (0.11) genetic distance was recorded between genotypes 36550 and 36567, 36605 and 36592, 30881 and 36580, and 30881 and 36587.

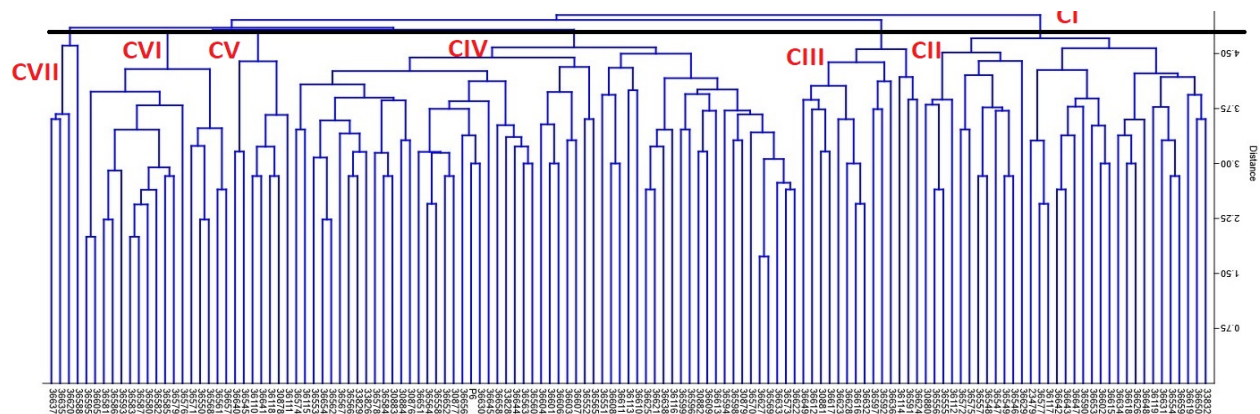
Cluster analysis on the basis of SSR markers

Cluster analysis was performed to assess genetic diversity in *Capsicum* germplasm using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on a similarity matrix (Fig. 5). The dendrogram provided further validation of the relationships between the genotypes. The analysis revealed that 133 genotypes were classified into seven distinct clusters at a linkage distance of 5.0.

In this analysis, Cluster I (CI) included 21 genotypes, representing 15.7% of the total variation observed (Table 3). The genotypes grouped in this cluster were 33832, 36650, 36619, 36559, 36554, 36631, 36119, 36648, 36626, 36618,

Table 3: Frequency of *Capsicum annum* genotypes for SSR based on dendrogram

Cluster	Frequency	Genotypes of (<i>Capsicum annum</i>)
CI	21	33832, 36650, 36619, 36559, 36554, 36631, 36119, 36648, 36626, 36618, 36634, 36615, 36602, 36653, 36590, 36647, 36643, 36642, 36117, 36577 and 23479.
CII	12	36629, 36546, 36549, 36547, 36548, 36557, 36575, 36572, 36112, 36555, 36556 and 30880.
CIII	13	36624, 36591, 36114, 36636, 36592, 36632, 36616, 36628, 36623, 36617, 30881, 36612 and 36649.
CIV	58	36622, 36573, 36633, 36569, 36627, 36570, 30879, 36598, 36594, 36613, 36609, 30882, 36596, 36599, 36116, 36638, 36621, 36625, 36610, 36113, 36608, 36551, 36565, 36552, 36607, 36603, 36606, 36601, 36604, 36560, 36563, 36644, 33828, 36658, 36645, 36630, P6, 36656, 308877, 36652, 36558, 36564, 36651, 30876, 30884, 30883, 36584, 36578, 33826, 33829, 36566, 36567, 36562, 36654, 36654, 35653, 36115 and 36574.
CV	7	36111, 30878, 36118, 36641, 36610, 36545 and 36640.
CVI	17	36657, 36561, 36568, 36550, 36571, 36576, 36579, 36585, 36582, 36580, 36587, 36583, 36593, 36586, 36581, 36605 and 36595.
CVII	6	366065, 36595, 36588, 36620, 36635 and 36637

**Fig. 5:** Dendrogram of *Capsicum* genotypes based on SSR markers

36634, 36615, 36602, 36653, 36590, 36647, 36643, 36642, 36117, 36577 and 23479. Genotypes grouped in the cluster originated from the different geographical zones (Fig. 1). Genotype 36619 was identified as an outlier within the cluster due to its relatively high maximum genetic distance, recorded at (4.0). A minimum (2.50) genetic distance within the cluster was observed between genotypes 36643 and 36642.

Cluster II (CII) contained 12 genotypes, accounting for 9.2% of the total genetic variation in the analysis. The genotypes included were 36629, 36546, 36549, 36547, 36548, 36557, 36575, 36572, 36112, 36555, 36556 and 30880. Genotype 36629 was identified as an outlier due to its high (4.35) genetic distance. This may suggest unique alleles or genetic traits that differentiate it from the other genotypes. The lowest (1.75) genetic distance within the cluster was observed between genotypes 36555 and 36556.

Cluster III (CIII) consisted of 13 genotypes, representing 9.77% of the total genetic variability observed. The genotypes included in this cluster were 36624, 36591, 36114, 36636, 36592, 36632, 36616, 36628, 36623, 36617, 30881, 36612 and 36649. Among these, genotype 36114 was identified as an outlier due to its significantly higher (4.25) genetic distance, whereas the lowest (2.35) genetic distance within the cluster was observed between genotypes 36632 and 36616.

Cluster IV (CIV) comprised 57 genotypes, accounting for total genetic variation (42.85%). The genotypes included in this cluster were 36622, 36573, 36633, 36569, 36627,

36570, 30879, 36598, 36594, 36613, 36609, 30882, 36596, 35699, 36116, 36638, 36621, 36625, 36610, 36113, 36608, 36551, 36565, 36552, 36607, 36603, 36606, 36601, 36604, 36560, 36563, 36644, 33828, 36658, 36645, 36630, P6, 36656, 308877, 36652, 36558, 36564, 36651, 30876, 30884, 30883, 36584, 36578, 33826, 33829, 36566, 36567, 36562, 36654, 36654, 35653, 36115 and 36574. Within CIV, the smallest (1.75) genetic distance was observed between genotypes 36599 and 36627.

Cluster V (CV) included 7 genotypes, representing a genetic variation of 5.2% in the gene pool. The genotypes in this cluster were 36111, 30878, 36118, 36641, 36610, 36545 and 36640. Genotype 36111 was identified as an outlier within this cluster due to its maximum (3.08) genetic distance. The minimum 2.90 genetic distance was found between genotypes 36641 and 36610, as well as between 30878 and 36118.

Cluster VI (CVI) comprised 17 genotypes, accounting for 12.78% of the genetic variability in the gene pool. The genotypes included were 36657, 36561, 36568, 36550, 36571, 36576, 36579, 36585, 36582, 36580, 36587, 36583, 36593, 36586, 36581, 36605 and 36595. Genotype 36576 was identified as an outlier in this cluster with a genetic distance of 4.0. The smallest (1.90) genetic distance was obtained between genotypes 36583 and 36587, as well as between 36595 and 36605.

Cluster VII (CVII) included 6 genotypes, representing 4.51% of the total genetic variability. The genotypes in this cluster were 366065, 36595, 36588, 36620, 36635 and

36637. Genotype 36588 was identified as an outlier due to its high (4.75) genetic distance, indicating significant genetic divergence from the other members of CVII. The smallest (3.40) genetic distance within CVII was seen between genotypes 36635 and 36637. The presence of a highly divergent outlier alongside closely related genotypes in CVII highlights the genetic variability within this cluster, providing valuable insights for potential breeding and genetic improvement efforts.

Discussion

A genetic diversity study is crucial as it plays an important role in the survival and adaptability of species (Begna and Begna 2021). When plants face environmental challenges, gene variations are important for their survival and adaptation. Morphological traits have long been the source of studying taxonomy and variability among populations and species (Rouhan and Gaudeul 2021). The introduction of DNA primer technology in the 1980s accelerated the understanding of genetic structure and variation, offering a range of genetic markers that are not influenced by environmental conditions, allowing for the tracking of important agronomic traits (Çakir 2023; Tripodi 2023).

Microsatellites, or simple sequence repeats (SSRs), are among the most extensively utilized DNA markers in plant molecular genetics due to their broad applicability in genome mapping, varietal identification, and genetic diversity assessments. The first report on the use of SSRs in plant species was published by Akkaya *et al.* (1992), who identified polymorphic SSR loci in soybean and demonstrated their potential as PCR-based molecular markers. This pioneering work laid the foundation for the widespread adoption of SSRs across diverse plant genomes. SSR markers are considered highly efficient due to their high level of polymorphism, even among closely related genotypes, making them ideal for distinguishing fine-scale genetic variation (Turudić *et al.* 2024). Asri *et al.* (2024) highlighted that SSRs require only small quantities of DNA, are easily transferable between laboratories, and are suitable for automation, facilitating high-throughput genotyping. These markers are PCR-based, and their reproducibility, co-dominant inheritance, and multi-allelic nature have enabled their development across numerous plant species (Alves *et al.* 2024). Furthermore, SSRs have demonstrated significant utility in evaluating phylogenetic relationships, assessing genetic variation within germplasm collections, and supporting applications such as gene cloning, functional gene tagging, detection of duplication events, and marker-assisted selection in breeding programs (Kundu *et al.* 2024).

In the present study, the genetic diversity among *Capsicum annuum* genotypes was assessed using simple sequence repeat (SSR) markers. A total of 40 SSR primers were initially selected based on previous reports of their polymorphic potential. Effective diversity analysis requires the use of markers that are polymorphic, uniformly

distributed across the genome, and capable of producing clear and interpretable amplification patterns. However, certain limitations are inherent to SSR analysis, including the occurrence of stutter bands, minor PCR products that can obscure accurate scoring, and the presence of null alleles. Additionally, the analysis of microsatellite markers can be complicated by stuttering effects. The presence of null alleles, which occur when SSR markers fail to bind due to sequence variations in the DNA, further complicates interpretation (Gupta and Singla 2024).

Microsatellite variation was measured by calculating the total number of alleles detected and the average number of alleles per locus. Polymorphism information content (PIC) values were used to quantify allelic diversity at each locus, providing insight into the informativeness of each marker (Kanaka *et al.* 2023). Allelic data were recorded in binary format, with the presence and absence of bands scored as '1' and '0', respectively. In this study, a total of 2,504 bands were scored across all markers, representing 72 alleles with an average of 2.48 alleles per locus. This average allele number is higher than the mean of 2.28 alleles per locus documented by Dantas *et al.* (2022), yet lower than 4.08 alleles per locus reported by Sood *et al.* (2023) in their assessment of 24 genotypes. Comparatively, Rabuma *et al.* (2020) reported an average of 2.2 alleles among 32 pepper accessions from Ethiopia and India using 14 SSR markers, while Woubit *et al.* (2021) observed a markedly higher mean of 8.54 alleles in 75 Ethiopian pepper genotypes analyzed with 13 SSR markers. Additionally, Christov *et al.* (2021) identified an average of 6.9 alleles per locus within a diverse collection of 179 *Capsicum annuum* accessions from six countries using 21 SSR markers. Dhaliwal *et al.* (2014) reported moderate allelic diversity with a mean of 2.78 alleles per locus and a maximum of four alleles amplified by the primer AVRDC PP 32. These variations emphasize the significant influence of genetic background and SSR marker selection on allele detection in *Capsicum* germplasm.

The polymorphism information content (PIC) serves as a measure of a marker's ability to detect genetic variation, considering both the number of alleles at a locus and their relative frequencies (Sadeghi *et al.* 2024). In the present investigation, PIC values among the SSR markers ranged from 0.098 to 0.992, with a mean value of 0.708 (Table 2). Of the 29 polymorphic markers analyzed, 22 exhibited PIC values greater than 0.5, indicating a high level of informativeness. Markers such as AVRDC 17, AVRDC 174, Agi96, HPMS 1-5, AVRDC 67, CAMS 311 and HPMS-148 recorded the highest PIC values, demonstrating their ability to capture a broad spectrum of genetic diversity. In contrast to the present findings, Sood *et al.* (2023) reported a narrower PIC range (0.14 to 0.76) with a mean of 0.54. Similarly, Guzman *et al.* (2020) observed a high average PIC value (0.775). The PIC values obtained in this study were higher than those reported in earlier studies, indicating broader marker informativeness. Such markers are particularly valuable in breeding programs, as they

enable the selection of genetically distinct parental lines and contribute to the conservation of allelic variation within germplasm collections.

Conversely, six SSR markers displayed PIC values below 0.5, suggesting limited discriminative power. Among them, CAMS-555 Bd70 showed the lowest PIC value (0.098), indicating low allelic diversity and reduced utility for differentiating closely related genotypes. Generally, lower PIC values may reflect a narrow genetic base among the accessions being studied, whereas higher values are indicative of greater allelic richness and more uniform allele distribution (Guha *et al.* 2025). Senior *et al.* (1998) reported that markers exhibiting a high number of alleles with balanced allele frequencies tend to produce the highest PIC scores. Furthermore, the informativeness of a marker system also depends on whether the markers are dominant or co-dominant, which affects the estimation of gene diversity (Bidyandana *et al.* 2024). Differences in observed polymorphism levels across studies may stem from variations in population structure, the genetic makeup of the germplasm, and the molecular methodologies employed (Dhaliwal *et al.* 2014).

Expected heterozygosity (H_e), a measure of genetic variability, was calculated for the 29 polymorphic SSR markers. It reflects the probability that two alleles randomly drawn from a given population will be different (Powell *et al.* 1996). Among the markers analyzed, HPMS 1-214 exhibited the highest H_e value, indicating a substantial degree of allelic diversity at this locus. This marker amplified five alleles, contributing to its elevated heterozygosity. However, despite its high H_e , HPMS 1-214 was associated with a moderate (PIC) value. This discrepancy may be attributed to unequal allele frequencies; while the presence of multiple alleles enhances heterozygosity, the PIC value is sensitive to the evenness of allele distribution. A marker with uneven allele frequencies may exhibit high heterozygosity but limited discriminatory capacity, as reflected by a lower PIC value. Thus, although HPMS 1-214 effectively captures genetic variation, its utility in distinguishing closely related genotypes may be inferior to markers with more balanced allele distributions and higher PIC values. This distinction underscores the complementary roles of H_e and PIC in evaluating marker informativeness (Nagy *et al.* 2022). In our study, (H_e) ranged from 0.038 to 0.766, with an average of 0.428. In contrast to our findings, Christov *et al.* (2021) reported a higher genetic diversity $H_e = 0.531$ with an average of 0.952 in *C. annuum* accessions.

In our study, SSR marker analysis revealed a high level of polymorphism, with 72.5% of the markers displaying allelic variation among the *Capsicum annuum* genotypes evaluated. This level of polymorphism is notably higher than that reported in several earlier investigations, highlighting substantial variation in the detection of genetic diversity across studies. For example, Zhong *et al.* (2021) identified 13.8% polymorphic markers from 240 SSR loci,

tested on 147 pepper cultivars. Similarly, Li *et al.* (2018) obtained 11.2%, Liu *et al.* (2014) reported 14.1%, and Wu *et al.* (2017) found 42.5% polymorphism. Dhaliwal *et al.* (2014) reported 54% polymorphism using 27 SSR markers across 64 genotypes, a value significantly lower than that observed in the current study. The reduced levels of polymorphism observed in these earlier studies may be attributed to differences in the genetic composition of the sampled genotypes, the number and selection of SSR primers, the resolution of the detection system and the overall genetic diversity within the studied populations. Furthermore, discrepancies in observed polymorphism levels may also result from variations in molecular methodologies, amplification conditions, or the genomic regions targeted by the primers. These findings underscore the importance of marker selection, population structure, and experimental design in assessing genetic variability through molecular methodologies. Genetic diversity within *Capsicum* populations varies considerably and is influenced by factors such as geographic origin, breeding history, and selection pressures (Fan *et al.* 2022). The effectiveness of SSR markers in detecting polymorphism is contingent upon their genomic distribution and their capacity to capture a wide array of allelic variants (Han *et al.* 2024). Markers that are evenly dispersed throughout the genome and capable of identifying multiple alleles tend to provide a more comprehensive assessment of genetic variation within populations.

Genetic diversity was quantified using the method described by Nei and Li (1979), which estimates genetic variation within a population. The greatest genetic distances were observed between the genotype pairs 36569 and 36627, 36616 and 36632, 36616 and 36628, and 36628 and 36632, indicating substantial genetic divergence among these genotypes. Conversely, the smallest genetic distances were detected between genotypes 36550 and 36567, 36605 and 36592, 30881 and 36580, as well as 30881 and 36587, reflecting a closer genetic relationship among these pairs. The pronounced genetic differentiation among certain genotypes suggests their potential utility as parental lines in breeding programs. Incorporating these genetically diverse genotypes can enhance the genetic base of breeding populations, thereby facilitating the development of improved cultivars with greater adaptability and trait variability.

The UPGMA cluster analysis of 133 *Capsicum* genotypes revealed substantial genetic diversity, organizing the genotypes into seven distinct clusters characterized by varying degrees of genetic similarity. Larger clusters comprised genotypes exhibiting close genetic relationships, whereas smaller clusters and isolated outliers represented genetically unique genotypes with potential value for breeding and conservation efforts. Notably, genetically divergent genotypes such as 36619, 36629, 36114 and 36588 may serve as important reservoirs of novel alleles for crop improvement programs. In contrast, genotype pairs

with minimal genetic distances, including 36555 and 36556 as well as 36599 and 36627, are likely to contribute less to heterosis but could play a role in maintaining genetic uniformity within breeding lines. The observed close genetic affinities between certain genotypes likely reflect shared ancestry or exposure to similar selective pressures, resulting in high genetic similarity (Voss-Fels *et al.* 2015).

The large Cluster IV (CIV) indicates a degree of genetic redundancy, suggesting that several genotypes within this group exhibit high similarity and may contribute limited additional value to overall genetic diversity. Conversely, clusters characterized by substantial genetic distances represent heterogeneous gene pools that are critical to conserve, as they provide essential variability to support future breeding initiatives and enhance adaptability to diverse environmental conditions. The identification of outlier genotypes with distinct genetic divergence highlights the presence of unique alleles, which can be strategically utilized in the development of cultivars with improved stress tolerance or enhanced yield potential (Vasumathy *et al.* 2021). Additionally, the detection of closely related genotypes within the collection may signify duplication, underscoring the need for careful management of germplasm resources to optimize genetic diversity.

Conclusion

Genetic diversity among 133 genotypes of *Capsicum annuum* was assessed using SSR markers to evaluate allele variability and their potential applicability in breeding programs. The analysis revealed a high level of polymorphism, with 72.5% of the markers showing considerable variability. This genetic variation was further confirmed through UPGMA-based cluster analysis, which grouped the genotypes into seven distinct clusters, indicating substantial genetic differentiation. The genetic distance matrix also demonstrated significant variability, identifying both genetically distinct and closely related genotypes. Genotypes such as 36569 and 36627, 36616, 36632 and 36628 exhibited high genetic distances, suggesting their potential utility in the development of improved cultivars with traits such as stress tolerance, high yield, and disease resistance. The study emphasizes the effectiveness of SSR markers in assessing genetic diversity and their capacity to detect a broad spectrum of alleles relevant for breeding. Markers like AVRDC 17, AVRDC 174, Agi96, HPMS 1-5 and CAMS 311, which recorded high PIC values, are particularly valuable due to their ability to capture extensive genetic variation. Future studies should aim to broaden the marker set to include more SSRs and other molecular markers for a more comprehensive assessment of genetic diversity in *Capsicum*. Investigating the genetic basis of agronomic traits such as disease resistance and environmental adaptation would further contribute to breeding advancements. In conclusion, the findings provide essential insights into the genetic structure

of *Capsicum annuum*, and the identified genotypes and informative markers offer a strong foundation for future breeding and conservation strategies.

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Authors Contributions

The study was conceived and designed by all authors. NK conducted the experiments and prepared the materials. Data analysis was carried out by NK, KK, SHK and SB. NK also drafted the initial version of the manuscript. MJA and SIA provided supervision and critical feedback throughout the research process. All authors reviewed, revised, and approved the final version of the manuscript.

Conflict of Interest

The author has no competing interests to declare.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

This study did not involve human participants or animals; therefore, ethical approval was not required.

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