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MOLECULAR CHARACTERIZATION AND GENETIC RELATIONSHIPS AMONG SUMMER SQUASH (*CUCURBITA PEPO* L.) GENOTYPES VIA SSR PROFILING

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ABSTRACT

Genetic diversity is a crucial prerequisite for effective crop improvement and the development of high-yielding, climate-resilient cultivars of summer squash (*Cucurbita pepo* L.). The present study assessed molecular genetic variability and genetic relationships among forty diverse summer squash genotypes using simple sequence repeat (SSR) markers. Genomic DNA extracted from young leaf tissues was amplified with eighteen SSR primer pairs, generating reproducible and polymorphic banding patterns. A total of thirty-nine alleles were detected across all loci, with alleles per locus ranging from one to three and an average of 2.16, indicating moderate allelic diversity within the germplasm. Polymorphic information content (PIC) values varied from 0.0476 to 0.3693, with a mean of 0.2977, reflecting the moderate to high informativeness of the SSR markers. Cluster analysis based on genetic distance grouped the genotypes into three major clusters, revealing substantial genetic differentiation. While several genotypes showed close genetic similarity, distinct accessions such as SKUA-SQ-10 and SKUA-SQ-20 exhibited marked genetic divergence, highlighting their potential utility as parental lines in breeding programmes. The clustering pattern showed no strict association with genotype origin, suggesting historical gene flow and shared ancestry. Overall, the study confirms the effectiveness of SSR markers for genetic diversity assessment and provides valuable molecular insights to support germplasm conservation, parental selection and strategic hybridization for genetic improvement of summer squash.

KEYWORDS: *Cucurbita pepo* L.; Simple sequence repeat (SSR) markers; polymorphic information content; cluster analysis.

INTRODUCTION

Cucurbita pepo L. is the botanical designation for summer squash, a horticultural crop cultivated during the summer with chromosome number **2n=2x=40**. Several traditional denominations for squash, a monoecious vegetable species, include *baby marrow* (South Africa), *courgette* (America), *marrow* (Ireland and Britain), *cucuzza* (Saudi Arabia) and *zucchini* (Italy). According to **Tadmor *et al.* (2005)**, the squash (*Cucurbita pepo L.*), one of the most archaic genera (*Cucurbita*) in the botanical world, is an economically vital taxon of the **Cucurbitaceae** family. Indigenous to the eastern United States and Mexico, it is cultivated in temperate, subtropical and tropical agro-climates and is extensively grown for its fruits worldwide (**Bisognin, 2002**). *Cucurbita pepo* is among the most **polymorphic plant taxa** and possesses perhaps the most polymorphic fruit morphology among all Cucurbitaceae members. Squash is generally termed **"summer squash"** while in its juvenile stage. Because of its delicate and immature fruits, which are typically consumed when the pericarp is smooth and lustrous and the seeds remain undeveloped, it is cultivated commercially across the globe. Due to its abbreviated growth duration and elevated productivity, the fruit attains maturity **40–50 days post-seeding**. There are two growth habits: bush and vining types of summer squash. The closely set fruits of bush squashes, sometimes referred to as summer squash, are ready for harvest within six weeks, and their stems exhibit considerably shorter internodes. The vines produced by these genotypes attain several meters in length and attach to surfaces through tendrils. Typically, these vines bear large, lobed foliage. In vining squash, annual cultivars complete their life cycle within a single growing season.

According to **Thamburaj and Singh (2001)**, summer squash is also referred to as bush squash, vegetable marrow, zucchini, *Vilayati Kaddu*, *Chappan Kaddu* and *Safed Kaddu*. The wide array of phytochemicals, nutrients, vitamins and minerals constituting its nutritional composition are responsible for its remarkable health benefits (**Gramza-Michałowska and Kulczyński, 2019**). It is particularly rich in bioactive constituents and essential nutrients including phenolics, flavonoids, vitamins, amino acids, carbohydrates and minerals—especially potassium. It also contains high dietary fiber and a low caloric value (approximately 17 kcal/100 g of fresh fruit) (**Tamer *et al.*, 2010**). Numerous pharmacological effects have been documented, including antidiabetic, antihypertensive, antitumor, antimutagenic, immunomodulatory, antibacterial, antihypercholesterolemic, intestinal antiparasitic and anti-inflammatory activities, along with potential applications of other *Cucurbitaceae* species (**Kostalova *et al.*, 2009**). Due to its low caloric density, it is also regarded as a dietary component for weight management (**Fageria *et al.*, 2012**). Therefore, one of the key strategies to ensure food and nutritional security is to enhance global production while recognizing its significance.

According to **Balluz *et al.* (2005)**, squash is a valuable nutritional source aiding in the prevention of cancer, cardiovascular disease and inflammatory disorders such as arthritis and asthma, owing to its content of vitamins A, C, niacin, folate and dietary fiber. Asia accounts for nearly half of the global land area devoted to squash and pumpkin cultivation (**Al Brifcany, 2015**). As per the **National Horticulture Board (NHB, 2023–2024)**, squash and pumpkin production in India reached 2,703.61 thousand MT annually. In the Kashmir region, summer squash is cultivated over approximately 3,200 hectares, producing an estimated 64,800 tonnes (**Anonymous, 2024**). Cultivated widely across India, this cucurbit is fast-growing, early-yielding and performs optimally under moderate, humid climatic conditions, requiring a temperature range of 16–27 °C for proper growth and development.

Prior research endeavors attempted to investigate the genetic performance of squash in various environmental settings (Swain *et al.*, 2022; Sajid *et al.*, 2022). However, not much is known about the genetic divergence and variability

linked to yield-contributing and quality parameters in squash accessions. Therefore, it is imperative to assess the current summer squash germplasm in order to identify superior genotypes with great potential in terms of quality and yield characteristics. Producing such data could help create climate-resilient cultivars with improved yield and quality.

The widespread use of molecular markers has made it possible to understand the changes throughout time and the interactions between genetically diverse genes (Chakravarthi and Naravaneni 2006; Yuan *et al.,* 2015). (Singh *et al.,* 2016) claim that molecular genetics has advanced significantly in the identification and application of molecular markers to detect and take advantage of DNA polymorphisms. Since SSRs are multiallelic, highly repeatable, co-dominantly inherited and have a large genome coverage, microsatellites are a useful tool for researching them according to (Kumar *et al.,* 2019). SSR markers are helpful in determining different genotypes or those associated with important agronomic traits in a number of plant species among other traits. The identification, classification and genotyping of plants within a germplasm collection were studied by (Mady *et al.,* 2013). Using Random Amplified Polymorphic DNA (RAPD) as a molecular method this study evaluated the relationships and diversity among 20 landraces of summer squash (*Curcubita pepo* L.) which are historically used to treat prostate hyperplasia and hypertension. Of the 65 reproducible bands that were produced by ten RAPD primers, 46 (70.77%) were polymorphic, suggesting that the summer squash lines had a wide variety of genotypes. Using cluster analysis, the summer squash germplasm was separated into two groups: one with one landrace and another with 19 landraces that could be further subdivided into five groups. The study's findings suggest that RAPD markers may be used to identify and evaluate genetic differences between squash landraces. They also offer several options for creating an effective breeding program to enhance summer squash.

MATERIAL AND METHODS

Plant materials

During Kharif-2024, the yield and yield-attributing traits of forty genotypes of summer squash with phenotypically different characteristics that were gathered from various sources were evaluated. In Table 1, the genotypes and their corresponding sources are displayed. Seed sowing was carried out on April 23, 2024, at the Vegetable Experimental Farm, Division of Vegetable Science, SKUAST Kashmir, Shalimar Srinagar.

DNA isolation and quantification

DNA was extracted from young, fresh leaves that were devoid of insects and diseases across all 40 genotypes. Leaf samples were taken when the plants were still seedlings. Prior to DNA extraction and SSR marker analysis at the Division of Plant Breeding and Genetics, SKUAST-K, the samples were kept in a deep freezer at -80˚C. For DNA extraction, one gram of leaf tissue was homogenized in 700 µl of CTAB extraction buffer using a mortar and pestle to ensure efficient cell disruption. The homogenate was incubated at 65°C for one hour with periodic inversion of the tubes to maintain thorough mixing. Following incubation, 700 µl of chloroform: isoamyl alcohol (24:1) was added and phase separation was achieved by centrifugation at 12,000–14,000 rpm for 20 minutes at 4°C. The resulting aqueous phase was carefully transferred to a fresh 1.5 ml tube and DNA was precipitated by adding 500 µl of cold isopropanol.

Samples were stored overnight at –20°C to enhance precipitation and subsequently centrifuged at 12,000–14,000 rpm for 20 minutes at 4°C. The DNA pellet was washed with 700 µl of 70% ethanol, followed by an additional centrifugation at 10,000 rpm for 10 minutes. After ethanol removal, the pellet was air-dried under laminar airflow and finally dissolved in 50 µl of TE buffer or nuclease-free water. DNA concentration and purity were evaluated through

agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving 0.5 g of agarose in 50 ml of 1X TAE buffer (Tris base 45 mM, acetic acid 45 mM, EDTA 1 mM). The solution was microwaved until completely transparent, then allowed to cool slightly before being stained with 2 µl of ethidium bromide. The molten agarose was poured into a casting tray containing combs and left to solidify at room temperature for 20–25 minutes. For electrophoresis, each DNA sample (3 µl) was mixed with 1 µl of loading dye and carefully loaded into the wells. The gel was run at 80 V for approximately two hours, after which the DNA bands were visualized under UV light to assess integrity and overall yield.

Table 1: List of summer squash (*Cucurbita pepo* L.) genotypes used in the present study.

S. No.	Genotype Name	Source
1	SKUA-SQ-1	COLLECTION
2	SKUA-SQ-2	COLLECTION
3	SKUA-SQ-3	COLLECTION
4	SKUA-SQ-4	COLLECTION
5	SKUA-SQ-5	COLLECTION
6	SKUA-SQ-6	COLLECTION
7	SKUA-SQ-7	COLLECTION
8	SKUA-SQ-8	COLLECTION
9	SKUA-SQ-9	COLLECTION
10	SKUA-SQ-10	COLLECTION
11	SKUA-SQ-11	Private company
12	SKUA-SQ-12	Private company
13	SKUA-SQ-13	COLLECTION
14	SKUA-SQ-14	Private company
15	SKUA-SQ-15	Private company
16	SKUA-SQ-16	IIHR
17	SKUA-SQ-17	Private company
18	SKUA-SQ-18	Private company
19	SKUA-SQ-19	IIVR
20	SKUA-SQ-20	IIVR
21	SKUA-SQ-21	COLLECTION
22	SKUA-SQ-22	IIVR
23	SKUA-SQ-23	IIVR
24	SKUA-SQ-24	IIVR
25	SKUA-SQ-25	IIVR
26	SKUA-SQ-26	IIVR
27	SKUA-SQ-27	IIVR
28	SKUA-SQ-28	IIVR
29	SKUA-SQ-29	NBPGR
30	SKUA-SQ-30	IIVR
31	SKUA-SQ-31	COLLECTION
32	SKUA-SQ-32	COLLECTION
33	SKUA-SQ-33	COLLECTION
34	SKUA-SQ-34	COLLECTION
35	SKUA-SQ-35	COLLECTION
36	SKUA-SQ-36	COLLECTION
37	SKUA-SQ-37	COLLECTION
38	SKUA-SQ-38	COLLECTION
39	SKUA-SQ-39	COLLECTION
40	SKUA-SQ-40	COLLECTION

PCR amplification of DNA and electrophoresis

PCR amplification was carried out in a 0.2-mL PCR tube with a reaction volume of 12.5 µL, containing 3.25 µl, Forward primer (10µM) 1 µl, Reverse primer (10µM) 1 µl, GoTaq 6.251 µl, DNA sample 1 µl . The tubes were placed in an Eppendorf gradient master cycler programmed for initial denaturation at 94°C for 4 min followed by 35 cycles of 1 min at 94°C, 45 s at 50-60 °C, 1 min at 72°C and a final extension of 7 min at 72°C. One microliters of ethidium bromide were used to stain a 1.5% agarose gel, which was used to segregate the amplified SSR primer products. A molecular filter was created by the agarose gel's tiny holes. The electrophoresis running buffer was a 1X TAE buffer.

Two microliters of a 50 bp ladder were added to each well as a marker and four microliters of the PCR product were added. The gel was examined under a UV lamp after three hours of electrophoresis at 80 volts.

Scoring of SSR allele profile

Based on the bands' locations in relation to the DNA ladder, SSR allele sizes were calculated. With reference to the 50 bp standard marker (Thermo Scientific), allele differences were measured by fragment size (bp) and bands were evaluated based on their relative mobility in the gel. Each primer's banding patterns were compared across all genotypes. A binary method was used for marker scoring, where 0 corresponded to no amplification and 1 to amplification.

RESULTS

SSR polymorphism

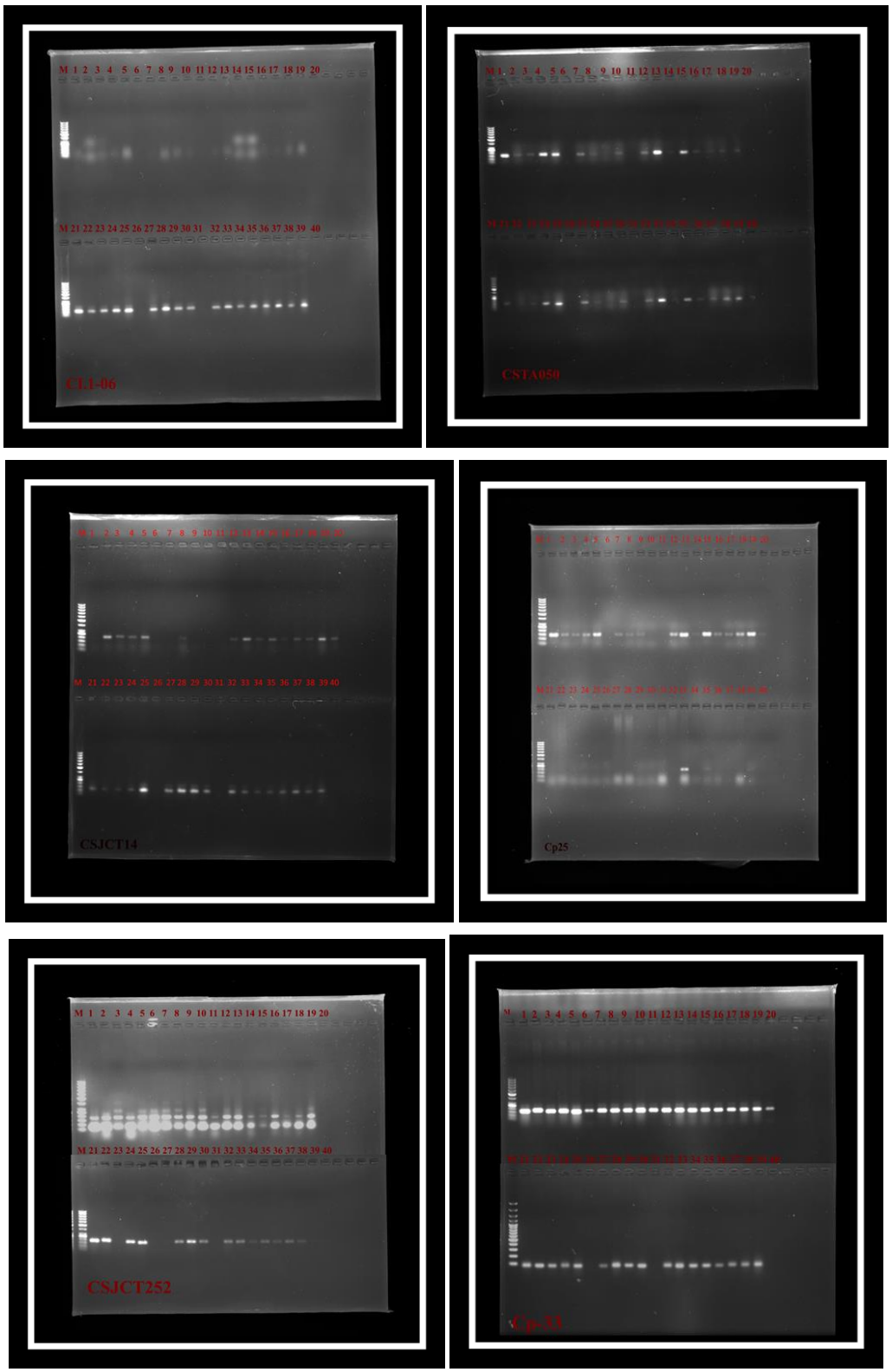
A set of 18 pairs of primers designed to amplify known SSR regions in summer squash were used to analyze genetic diversity of 40 summer squash varieties including local accessions and inbred lines.

Table 2: List of selected SSR markers along with their primer sequence.

S.No.	Marker	Forward Primer	Reverse Primer
1	CP-24	TGTAAAACGACGGCCGTGCTGCATGTTGGATGTCT	GTGACCATGGACAACACGTC
2	CP-33	TGTAAAACGACGGCCCTCTTCCGATTCTCCGCTTA	CCGATCAAGAACAGCACAGA
3	M121d	CCCAAATTCAAAAGAAAATC	CCAGTTAAATCAGACCGAAT
4	Cm22	TGTAAAACGACGGCCAGTCCAAAACCGACCAAATGTTCC	ATACAGACACGCCTTCCACC
5	CP-25	TGTAAAACGACGGCCCTCTTCCGATTCTCCGCTTA	TTCGAACTTGAGCAAGCAAA
6	CP-46	TGTAAAACGACGGCCTCTTCCGATTCTCCGCTTAG	GCACAGAAAACGGGGTAAAA
7	CP-56	TGTAAAACGACGGCCTCCATTTCCACTCATTTTTC	GATCCAGTTGAAGCGATTAC
8	CP-52	TGTAAAACGACGGCCTCACTTCTCCCCTTCTCTGC	TTCGAACTTGAGCAAGCAAA
9	CSTCC813	GTTGTGCTCCCCAATAGTTG	CACCACTTCTTCCACCGAA
10	CSJCT14	TTCCACGTTACATTGGACGA	AGAATTCATGGCCTGCAGAT
11	CSCCT571	CCTTTCTGCTGTTTCTTCTTC	CCTTTCTGCTGTTTCTTCTTC
12	CSTA050	GAATTATGCAGATGGGTCTT	CAAGAAGATCAAATGATAGC
13	CMTC51	ATTGGGGTTTCTTTGAGGTGA	CCATGTCTAAAAACTCATGTGG
14	CSJCT71	AATTCCATGGACATCCAGCCGAG	CAGTGAAAGGCACTAAAGCGGAG
15	CSJCT252	GATGGTGGAGATGGAATTGGGAT	TTAGAGCTGGAACTCTCCGCAAC
16	CI.1-06	CACCCTCCTCCAGTTGTCATTCG	AAGGTCAGCAAAGCGGCATAGG
17	CSJCT656	TCCTACAACTCAAAGGGCCAAC	GAAGTGGAGTGGAGTGGAGTGA
18	CSJCT662	ACGTCGTAAAACCATCGGAGTC	GCTTCCAAGCGTCAAAGGTATC

A total of 39 polymorphic alleles were identified across 18 SSR loci. The number of alleles per locus ranged from 1 to 3, with an average of 2.16 alleles per locus. The highest number of alleles (3) was observed for markers CP-24, CSJCT252, CSJCT656, CSJCT050 and CI.1-06, while the lowest (1) was recorded for marker M121-D and CSJCT71.

The effectiveness of a marker is determined by its ability to balance the detection of multiple alleles with varying levels of polymorphism (Powell *et al.,* 1996). The number of alleles per locus obtained in this study aligns with the findings of Sarao *et al.* (2014), Yildiz *et al.* (2015) and Mahapatra *et al.* (2022). Below fig shows banding pattern of the summer squash genotypes based on different SSR markers. The variation in allele numbers across loci reflects the heterozygosity at specific loci, indicating genetic variability within the population.



Genetic variation among varieties

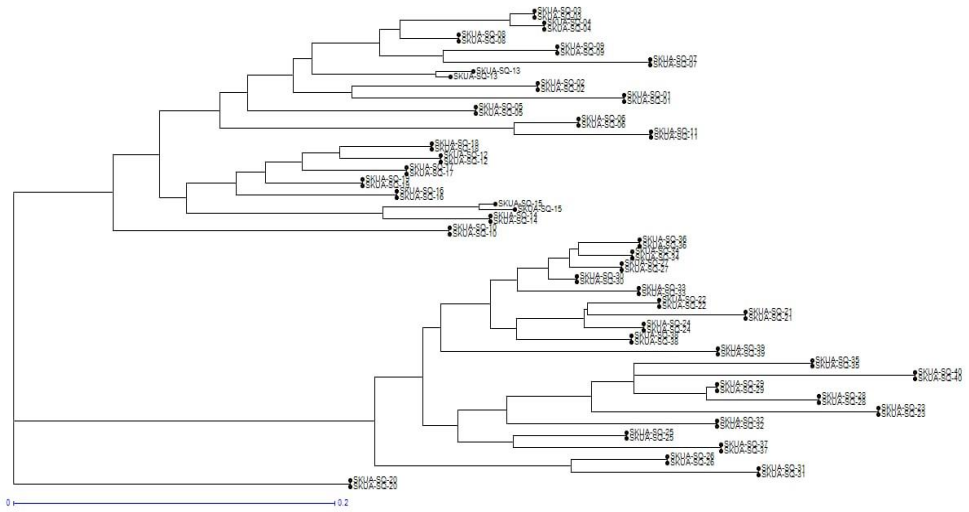
The Polymorphic Information Content (PIC) value is a robust measure of the discriminating ability of molecular markers, as it simultaneously accounts for both the number of alleles at a locus and their relative frequencies. Lower PIC values generally reflect limited genetic variation and close relatedness among genotypes, whereas higher values indicate greater allelic diversity and enhanced marker informativeness. As presented in Table 3, the 18 polymorphic SSR markers exhibited PIC values ranging from 0.0476 (CSJCT050) to 0.3693 (CP-33, CSJCT14 and CI.1-06), with a mean PIC value of 0.2977 across the 40 summer squash genotypes analyzed. This relatively high average PIC value clearly demonstrates the strong discriminatory power and high informativeness of the SSR markers employed in the present study. Comparable levels of marker polymorphism and informativeness have also been reported by Gürcan et al. (2015), Yildiz et al. (2015) and Mahapatra et al. (2022), thereby corroborating the reliability and effectiveness of SSR markers for assessing genetic diversity in summer squash.

Table 3: Allelic variation and polymorphic information content (PIC) for SSR loci across 40 selected Summer Squash (*Cucurbita pepo* L.) genotypes.

S. No	Primer	Number of alleles amplified	Expected fragment size(bp)	PIC Value
1	**M121-D**	1	50-100	0.3589
2	**CP-52**	2	100-200	0.3425
3	**CP-46**	2	100-200	0.3648
4	**CP-24**	3	100-300	0.3515
5	**CP-56**	2	150-250	0.2471
6	**CM-22**	2	150-250	0.2225
7	**CP-25**	2	100-250	0.3648
8	**CP-33**	2	50-200	0.3693
9	**CSCCT571**	2	100-200	0.3192
10	**CMTC51**	2	100-200	0.2879
11	**CSJCT71**	1	150-500	0.1948
12	**CSJCT252**	3	100-300	0.3648
13	**CSJCT656**	3	150-300	0.3318
14	**CSTCC813**	2	250-300	0.1638
15	**CSTA050**	3	100-250	0.0476
16	**CSJCT14**	2	100-250	0.3693
17	**CI.1-06**	3	100-250	0.3693
18	**CSJCT622**	2	100-200	0.2879
	Total	39		5.357
	Average	2.16		0.2977

Cluster analysis

The allelic diversity data were used to construct a dendrogram based on a genetic distance matrix, which elucidated the genetic relationships among the 40 summer squash genotypes evaluated (Fig. 1). Dendrogram analysis is a powerful and reliable approach for summarizing microsatellite data and resolving genetic relationships, even among closely related or phenotypically similar genotypes. The resulting dendrogram classified the genotypes into three major clusters (Table 4), reflecting substantial genetic differentiation within the germplasm.



Cluster I was further subdivided into two distinct sub-clusters, Ia and Ib. Sub-cluster Ia comprised 18 genotypes, namely SKUA-SQ-3, SKUA-SQ-4, SKUA-SQ-8, SKUA-SQ-9, SKUA-SQ-7, SKUA-SQ-13, SKUA-SQ-2, SKUA-SQ-1, SKUA-SQ-5, SKUA-SQ-6, SKUA-SQ-11, SKUA-SQ-18, SKUA-SQ-12, SKUA-SQ-17, SKUA-SQ-19, SKUA-SQ-16, SKUA-SQ-15 and SKUA-SQ-14, indicating a high degree of genetic relatedness among these accessions. Sub-cluster Ib was represented by a single genotype, SKUA-SQ-10, suggesting its genetic distinctiveness within Cluster I. Cluster II was also divided into two sub-clusters, IIa and IIb. Sub-cluster IIa included 18 genotypes—SKUA-SQ-36, SKUA-SQ-34, SKUA-SQ-27, SKUA-SQ-30, SKUA-SQ-33, SKUA-SQ-22, SKUA-SQ-21, SKUA-SQ-24, SKUA-SQ-38, SKUA-SQ-39, SKUA-SQ-35, SKUA-SQ-40, SKUA-SQ-29, SKUA-SQ-28, SKUA-SQ-23, SKUA-SQ-32, SKUA-SQ-25 and SKUA-SQ-37—highlighting considerable intra-cluster diversity. Sub-cluster IIb consisted of two genotypes, SKUA-SQ-26 and SKUA-SQ-31, which formed a separate subgroup within Cluster II, reflecting moderate genetic divergence. Cluster III contained a single genotype, SKUA-SQ-20, underscoring its pronounced genetic uniqueness relative to the remaining accessions. Overall, the clustering pattern clearly demonstrates the high efficiency and discriminatory power of SSR markers in differentiating summer squash genotypes and grouping them according to their genetic similarity. The observed clustering trends and levels of genetic variation are in strong agreement with earlier reports by Mashilo et al. (2016b), Bonthala et al. (2022) and Mahapatra et al. (2022). The dendrogram thus reveals substantial genetic diversity among the summer squash accessions in relation to the SSR primers employed in this study.

Table 4: Distribution of Summer Squash (*Cucurbita pepo* L.) genotypes into different clusters based on SSR marker analysis

Cluster number	Sub Cluster	Genotypes	Number of genotypes in each cluster
I	**Ia**	SKUA-SQ-3,SKUA-SQ-4,SKUA-SQ-8,SKUA-SQ-9,SKUA-SQ-7,SKUA-SQ-13,SKUA-SQ-2,SKUA-SQ-1,SKUA-SQ-5,SKUA-SQ-6,SKUA-SQ-11,SKUA-SQ-18,SKUA-SQ-12,SKUA-SQ-17,SKUA-SQ-19,SKUA-SQ-16,SKUA-SQ-15,SKUA-SQ-14	18
	Ib	SKUA-SQ-10	1

II	**IIa**	SKUA-SQ-36,SKUA-SQ-34,SKUA-SQ-27,SKUA-SQ-30,SKUA-SQ-33,SKUA-SQ-22,SKUA-SQ-21,SKUA-SQ-24,SKUA-SQ-38,SKUA-SQ-39,SKUA-SQ-35,SKUA-SQ-40,SKUA-SQ-29,SKUA-SQ-28,SKUA-SQ-23,SKUA-SQ-32, SKUA-SQ-25, SKUA-SQ-37	18
	IIb	SKUA-SQ-26, SKUA-SQ-31	2
III		SKUA-SQ-20	1

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