Molecular Characterization and Genetic Diversity Analysis in Marigold (Tagetes spp.) using EST-SSR, SSR and ISSR Markers

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Abstract

We studied genetic diversity and population structure in 19 marigold (*Tagetes* spp.) genotypes, including *Tagetes erecta* and *Tagetes patula*, using ISSR, EST-SSR, and SSR primers. Out of 94 primers, 44 ISSR, 16 EST-SSR, and 19 SSR produced polymorphic bands, with polymorphism rates of 93.39%, 83.13%, and 89.77%. The average polymorphic information content was 0.35 for ISSR and EST-SSR, and 0.31 for SSR. Bayesian population structure analysis identified two genetic groups (K = 2) corresponding to the two species, and both UPGMA clustering based on Jaccard's similarity coefficient and principal coordinate analysis showed the same pattern. *T. erecta* genotypes had higher Shannon diversity index (0.39 ± 0.01) and expected heterozygosity (0.26 ± 0.01) than *T. patula* (0.09 ± 0.01 and 0.06 ± 0.01). Banding pattern analysis revealed 489 bands with 191 private alleles in *T. erecta* and 366 bands with 68 private alleles in *T. patula*, confirming species-specific differences. Analysis of molecular variance (AMOVA) showed that 52% of genetic variation was between populations and 48% was within populations. These salient findings of novel primers will be of great use in marigold germplasm conservation, Characterization and molecular breeding programme.

Introduction

Marigold (*Tagetes* spp.) belongs to the Asteraceae family and is among the most widely cultivated ornamental flowering plants worldwide. There are about 50 species in this genus, most of which originated from Central and South America. The African marigold (*Tagetes erecta* L.) and French marigold (*Tagetes patula* L.) are the most widely grown. Marigold is known for its varied flower colour and shape, and they are used in gardens, landscaping, pot-culture, floral arrangements, garlands, and religious ceremonies. Besides its ornamental values, marigold flowers are an important source of carotenoids, especially lutein, which is used in the food, pharmaceutical, cosmetic, and poultry industries (Hadden et al., 1999).

Floriculture sector is growing rapidly in Indian agriculture, and marigold contributes a large part of the commercial flower market. In India, Marigold is cultivated in an area of 103.34 thousand hectares with production of 1,153.80 thousand metric tonnes (MoA&FW, 2024-25). Due to their economic importance, the demand for novel flower colours and flower farms, tolerance to stresses, and rich carotenoid content, varieties/hybrids with these traits are attracting increased focus for development. The success of any breeding programme depends on the availability of genetic diversity and variation. Therefore, the study of genetic diversity is a basic aspect of any breeding programme. Studying genetic diversity using phenotypic data is less reliable because these traits are influenced by environmental factors. Molecular markers offer a more consistent way to study genetic variation since they are not influenced by environmental factors (Fischer et al., 2017). Common marker systems for studying genetic diversity include simple sequence repeats (SSRs), expressed sequence tag-derived SSRs (EST-SSRs), inter-simple sequence repeats (ISSRs), Random Amplified Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP). SSR markers are highly variable, codominant, and give reliable results. EST-SSRs

come from coding regions and may be linked to important genes. ISSR markers are affordable and good for finding genetic differences across the genome (Varshney et al., 2005).

Although marigold is an important flower crop, only a few molecular markers have been reported, and few studies have explored their genetic diversity using them. This study aims to develop and use ISSR, EST-SSR, and SSR markers to assess genetic diversity in marigold genotypes. The results will help provide molecular markers for future work in germplasm conservation, germplasm Characterization, and breeding of marigold.

Materials and Method

Plant Material and DNA Extraction

Nineteen marigold genotypes (Table 1) were selected for this study and all were maintained at the ICAR-Directorate of Floricultural Research farm in Pune. Modified CTAB method were used for genomic DNA isolation. Young, 200–300 mg of leaf tissue was collected and transferred to a 1.5 mL Eppendorf tube containing 1 mL pre-warmed CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol). The tissue was ground, incubated in a water bath at 65°C for 40 min, and centrifuged at 10,000 rpm. The aqueous phase was extracted twice with chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 rpm and then treated with 0.75 mL chilled isopropanol and kept at -20°C overnight for precipitation. Precipitated DNA was pelleted by centrifugation at 13,000 rpm, washed twice with 70% ethanol, air-dried, and re-suspended in 50–100 µL TE buffer. DNA quality and concentration were assessed by 0.8% agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific). Samples with intact high–molecular-weight DNA and A260/280 ratios of 1.8–2.0 were diluted to 25 ng/µL for PCR.

Table 1
List of marigold genotypes used in this study with their species, breeding center and its capitulum type

Sr. No.	Genotypes	Species	Breeding Center
1	Arka Bhanu	*Tagetes erecta*	ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru
2	Arka Abhi	*Tagetes erecta*	ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru
3	KAU M-46	*Tagetes erecta*	Kerla Agricultural University
4	KAU M-2	*Tagetes erecta*	Kerla Agricultural University
5	Pusa Arpita	*Tagetes patula*	ICAR-Indian Agricultural Research Institute (IARI), New Delhi
6	Pusa Basanti Gainda (PBG)	*Tagetes erecta*	ICAR-Indian Agricultural Research Institute (IARI), New Delhi
7	Pusa Narangi Gainda (PNG)	*Tagetes erecta*	ICAR-Indian Agricultural Research Institute (IARI), New Delhi
8	KAU M-1	*Tagetes erecta*	Kerla Agricultural University
9	CGFM-2	*Tagetes patula*	Indira Gandhi Krishi Vishwavidyalaya, Raipur
10	Pusa Deep	*Tagetes patula*	ICAR-Indian Agricultural Research Institute (IARI), New Delhi
11	DFR M-12	*Tagetes erecta*	ICAR - Directorate of Floricultural Research (DFR), Pune
12	DFR M-26	*Tagetes erecta*	ICAR - Directorate of Floricultural Research (DFR), Pune
13	DFR OPP-1	*Tagetes erecta*	ICAR - Directorate of Floricultural Research (DFR), Pune
14	Arka Shubha	*Tagetes erecta*	ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru
15	DFR M-2	*Tagetes erecta*	ICAR - Directorate of Floricultural Research (DFR), Pune
16	DFR-OPP3	*Tagetes erecta*	ICAR - Directorate of Floricultural Research (DFR), Pune
17	DFR HM-1	*Tagetes erecta*	ICAR - Directorate of Floricultural Research (DFR), Pune

Sr. No.	Genotypes	Species	Breeding Center
18	Pusa Parv	*Tagetes patula*	ICAR-Indian Agricultural Research Institute (IARI), New Delhi
19	Pusa Utsav	*Tagetes patula*	ICAR-Indian Agricultural Research Institute (IARI), New Delhi

Marker Development and Primer Selection

Transcriptome data for *Tagetes erecta* (BioProject PRJNA562616) were downloaded from the NCBI SRA database (Zhang et al., 2020), were assembled and screened for simple sequence repeats (SSRs) using the MISA tool with default settings. Primer pairs flanking each SSR motif were designed with Primer3 (Untergasser et al., 2012). Twenty-five EST-SSR primer pairs were randomly chosen for synthesis and validation. In addition, 20 SSR primers from Whankaew et al., (2014) and 49 ISSR primers were included. All primers were synthesized by Eurofins Genomics India Pvt. Ltd.

PCR Amplification and Gel Electrophoresis

PCR amplifications were performed in 96-well thermal cyclers (Applied Biosystems VeritiPro) with 25 µL reaction volumes containing 2 µL genomic DNA (25 ng/µL), 2.5 µL 10× KAPA Taq buffer, 1.5 µL 25 mM MgCl2, 0.5 µL 25 mM dNTP mix, 1 µL each of forward and reverse primers (10 µM), 0.1 µL KAPA Taq polymerase (5 U/µL), and 16.4 µL nuclease-free water. Thermocycling conditions included an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, primer-specific annealing (Ta) for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. After PCR, 2 µL of 6× loading dye was added to each sample. Amplified products from EST-SSR and genomic SSR primers were separated on 3% agarose gels, while ISSR products were resolved on 1.8% agarose, both in 1× TAE buffer. Electrophoresis was conducted at 80 V for 2–3 h, using 50 bp and 100 bp DNA ladders for fragment size estimation. Gels visualised using a BioZen Gel Doc system (ProKEMI916) with ethidium bromide staining. Distinct, reproducible bands were scored as present (1) or absent (0) for each genotype and marker. Despite of being co-dominant EST-SSR and SSR were scored as dominant to maintain compatibility with ISSR markers (Pratihari *et al*., 2026)

Genetic diversity and population structure analysis

The binary data matrix was used to assess genetic similarity and diversity among genotypes. Dendrogram was constructed using the UPGMA method in NTSYSpc Version 2.10e software with the Jaccard's similarity coefficient. Genetic diversity indices, including number of alleles (Na), effective alleles (Ne), Shannon's information index (I), expected heterozygosity (He), unbiased heterozygosity (uHe), Principal coordinate analysis (PCoA), were calculated using GenAlEx v6.5. Polymorphism

information content (PIC) was determined per locus (Botstein et al., 1980), and marker informativeness (effective multiplex ratio, marker index, resolving power) was assessed as described by Prevost and Wilkinson (1999). Population structure was analyzed with STRUCTURE v2.3.4 using an admixture model. K values from 1 to 10 were tested with five replicates each, using 1,000 burn-in iterations and 10,000 MCMC iterations per run. The optimal number of clusters was identified using the ΔK method. Analysis of molecular variance (AMOVA) and the genetic differentiation coefficient (PhiPT) among subpopulations were calculated in **GenAlEx v6.5.**

The calculations were performed using the following formulas:

$$PIC = 2p(1-p)$$

Where,

p = frequency of band presence (1)

1 − *p* = frequency of band absence (0)

$$EMR = PIC \times Total\ number\ of\ bands$$

$$MI = EMR \times PIC$$

$$Rp = \sum_{i=1}^{n} Ibi$$

Where, $Ibi = 1 - 2|0.5 - pi|$

pi = frequency of band presence (1) for band *i*

n = total number of bands from that primer

Results

The present study was carried out to understand the genetic diveristy and population structure across 19 marigold genotypes comprising two species, namely *Tagetes erecta* and *Tagetes patula*, using three molecular markers, i.e., ISSR, EST-SSR, and SSR primers (Supplementary Table 1). While binary scoring of SSR and EST-SSR primers can limit the detection of allelic variation, earlier studies show that combining several marker types yields reliable estimates of genetic relationships. Combining different marker analyses is a common approach in plant diversity research (Pratihari *et al*., 2026). ISSR, EST-SSR and SSR markers revealed substantial genetic polymorphism and variability among marigold genotypes studied, thereby confirming their utility for genetic diversity analysis and genotype discrimination.

Marker Polymorphism and Informativeness

ISSR Markers

Using 44 polymorphic ISSR primers, we obtained 451 clear, reproducible bands, of which 418 were polymorphic. This resulted in a polymorphism rate of 93.39% (Table 2). The number of bands per primer varied from 1 to 25, and the percentage of polymorphic bands per primer ranged from 44.44% to 100%. Primer ISSR-855 produced the highest number of polymorphic bands (21) (Supplementary Table 2). On average, each primer generated 9.5 polymorphic bands. The PIC values ranged from 0.19 to 0.45, with a mean of 0.35. MI values ranged from 0.04 to 3.03 (mean 1.31). Rp values ranged from 0.21 to 11.58 (mean 4.99), and EMR values ranged from 0.19 to 8.52 (mean 3.63).

EST-SSR and SSR Markers

Sixteen EST-SSR primers produced 42 bands in total, with 35 of them being polymorphic, which corresponds to 83.13% polymorphism (Table 2). Each primer produced 1–6 bands, and the percentage of polymorphic bands per primer ranged from 50% to 100%. Primer DN142342_c0_g1_I1 produced the highest number of polymorphic bands (5) (Supplementary Table 2). On average, each primer generated 2.19 polymorphic bands. PIC values ranged from 0.10 to 0.46, with an average of 0.35. EMR values ranged from 0.10 to 2.39 (average 0.95), MI from 0.01 to 0.98 (average 0.35), and Rp from 0.11 to 2.95 (average 1.13).

Nineteen SSR primers produced 64 bands, of which 57 were polymorphic, yielding a polymorphism rate of 89.77% (Table 2). On average, each primer produced 3.0 polymorphic bands. Primer TE02 generated the most polymorphic bands (8). The average PIC value was 0.31. The mean values for MI, EMR, and Rp were 1.12, 0.40, and 1.43, respectively. These findings show that SSR markers are effective for detecting genetic variation in marigold genotypes.

Table 2
Genetic diversity parameter in EST-SSR, SSR and ISSR markers in 19 marigold genotypes

Sr. No.	Primer Type	Total Primers	TB	PB	MB	Mean PPB	Mean PIC	Mean EMR	Mean MI	Mean Rp
1	EST-SSR	16	42	35	7	83.13	0.35	0.95	0.35	1.13
2	SSR	19	64	57	7	89.77	0.31	1.12	0.4	1.43
3	ISSR	44	451	418	33	93.393	0.353	3.632	1.309	4.993

TB = Total number of bands, PB = Total number of Polymorphic bands, MB = Total number of Monomorphic bands, PPB = Per cent polymorphic bands, PIC = Polymorphic Information Content, EMR=Effective Multiplex Ratio, MI=Marker index, Rp=Resolving Power.

Population Structure and Genetic Diversity

Bayesian clustering analysis identified two genetic groups (K = 2), corresponding to the highest ΔK value (Fig. 1). This approach separated marigold genotypes into *Tagetes erecta* and *Tagetes patula*, revealing

clear genetic differences between the species. The two clusters show species-specific divergence and little mixing between genotypes.

Genetic diversity measures showed more differences between the two groups. *T. erecta* had higher numbers of observed and effective alleles (1.65 and 0.824) than T. patula (1.445 and 1.111), which means it has more allelic variation. Shannon's Information Index was also higher in *T. erecta* (0.391) than in *T. patula* (0.093). Expected heterozygosity was greater in *T. erecta* (0.260) than in *T. patula* (0.063) (Table 3). Banding pattern analysis recorded 489 bands with 191 private alleles in *T. erecta* and 366 bands with 68 private alleles in *T. patula*, confirming species-specific differences (Fig. 2). Analysis of molecular variance (AMOVA) showed that 52% of the total genetic variation was between populations and 48% within populations (Table 4). This high level of variation between populations shows strong genetic differences between *T. erecta* and *T. patula*. The significant PhiPT value (0.515; $p < 0.001$) also shows strong population structure and limited gene flow.

Table 3
Summary of population cluster diversity

Population		N	Na	Ne	I	He	uHe
Tagetes erecta	**Mean**	13.998	1.650	1.445	0.391	0.260	0.270
	SE	0.002	0.029	0.016	0.011	0.008	0.008
Tagetes patula	**Mean**	5.000	0.824	1.111	0.093	0.063	0.070
	SE	0.000	0.029	0.012	0.009	0.006	0.007

Na = No. of Different Alleles, Ne = No. of Effective Alleles = 1 / (p^2 + q^2), I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q)), He = Expected Heterozygosity = 2 * p * q, uHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He.

Table 4
Analysis of AMOVA within and among populations

Source	df	SS	MS	Est. Var.	%	PhiPT
Among Pops	1	581.459	581.459	69.979	52%	0.515
Within Pops	17	1119.014	65.824	65.824	48%	
Total	18	1700.474		135.803	100%	

Genetic Relationship Analysis Using UPGMA and PCoA

The UPGMA cluster analysis carried out with Jaccard's similarity coefficient based on combined primer data. The similarity coefficients ranged from 0.40 to 0.95. With a similarity cut-off of 0.65, the UPGMA dendrogram grouped *T. erecta* and *T. patula* genotypes into two clusters, each representing a separate population (Fig. 3). Cluster I comprised 14 *T. erecta* genotypes, with similarity values ranging from 0.49

to 0.79. In this group, Arka Bhanu and Arka Abhi were the most closely related, with a similarity coefficient of 0.79. Cluster II included five *T. patula* genotypes, with similarity coefficients ranging from 0.85 to 0.95. Pusa Parv and Pusa Utsav were the closest in this group, with a coefficient of 0.95.

Principal coordinate analysis (PCoA) also confirmed the genetic relationships among marigold genotypes (Fig. 4), classified the two species into different clusters, and the results are consistent with UPGMA clustering, supporting the reliability of the genetic groupings.

Discussion

ISSR markers proved to be highly efficient, with a polymorphism rate of 93.39%. This high rate is likely due to ISSR primers amplifying multiple microsatellite regions across the genome (Gemmill & Grierson, 2020). The average PIC value of 0.35 shows they can clearly distinguish between genotypes. Higher MI, EMR, and Rp values also show that ISSR markers are effective at detecting many polymorphic loci (Venkatesan *et al.*, 20 21). Primer ISSR-855 was the most informative, producing the most polymorphic bands. The high resolving power of ISSR markers makes them useful for distinguishing closely related genotypes (Namita et al., 2013). These results are similar to earlier studies on marigold. For example, one study with 22 genotypes and 19 ISSR primers identified 184 amplicons, of which 171 (92.73%) were polymorphic. The PIC ranged from 0.23 to 0.47 (average 0.34), RP from 7.00 to 16.00 (average 11.20), and MI from 0.77 to 4.83 (average 2.93) (Panwar et al., 2017). Another study with 15 marigold genotypes and 12 ISSR primers reported a mean polymorphism of 60.48% (range: 40.00%-90.00%), an average PIC of 0.207, an average Rp of 4.53, and an average marker index of 2.39 (Namita et al., 2013). The higher polymorphism and PIC in this study are likely due to the greater number of ISSR markers used compared with previous studies.

High polymorphism in SSR primers, supports their use in genetic diversity studies of marigold. Because they are locus-specific, and highly reproducible, SSRs are useful for identifying genotypes and studying populations (Tsykun et al., 2017). The average PIC value of 0.31 suggests they have a good ability to distinguish between genotypes. While EMR and Rp values were lower than those for ISSR markers, SSRs still gave reliable estimates of genetic polymorphism. Primer TE02 was especially useful, as it amplified the most polymorphic loci. The moderate MI values for SSR markers likely result from fewer loci amplified per primer than for ISSR markers.

EST-SSR markers showed lower polymorphism than ISSR markers, but they were effective for studying genetic diversity because they target expressed regions of the genome, which usually show less polymorphism, as coding regions are more conserved (Ponyared et al., 2016). Even though MI, EMR, and Rp values were lower, EST-SSR markers are valuable because they are linked to functional genes and can be used across related species (Jiang et al., 2020). Previous studies on marigold genetic diversity using 19 SSR markers found an average PIC value of 0.57 (Yan et al., 2026), and Lahkar et al., (2024) reported an average PIC value of 0.44 in marigold using 14 SSR markers. The lower PIC values for SSR and EST-SSR markers in this study are likely due to the use of binary scoring.

Earlier research found similar results, dividing 26 marigold genotypes into two main groups, separating *T. erecta* and *T. patula* using 38 simple sequence repeats (Whankaew et al., 2014). Other studies using 23 SSR RAPD primers and 12 ISSR primers on 15 marigold genotypes also grouped them into two clusters: *T. erecta* and *T. patula* (Namita et al., 2013). The greater diversity in *T. erecta* may be due to its broader genetic base, longer cultivation history, and frequent use in breeding. Our findings match a previous study of 89 marigold accessions, which only included *T. erecta* and reported a Shannon's index (I) of 0.54, an average observed heterozygosity (Ho) of 0.64, and an average expected heterozygosity (He) of 0.54. The lower diversity observed in this study may be due to differences in the germplasm used, marker systems, the number of loci studied, and the binary scoring method for dominant marker data. Also, using closely related breeding materials here may have reduced the amount of detectable variation compared to more diverse collections.

Private alleles matter because they show unique genomic regions that may affect traits, appearance, or breeding value (Debener, 2012). The higher number of private alleles in *T. erecta* supports its greater genetic diversity and wider variation (Namita et al., 2013). Nguyen *et al.*, found similar results, with 54.06% variation among populations and 45.94% within populations in marigold germplasm. These consistent findings show that molecular markers are effective for detecting genetic differences between Tagetes species. This result is similar to earlier studies by Namita et al., (2013) and Whankaew et al., (2014). They also found that cluster analysis separated these two species using different molecular markers. The clear separation of the two Tagetes species into distinct clusters shows that the combined marker system is effective for genetic diversity analysis and highlights the value of these molecular markers for estimating genetic diversity in breeding and germplasm conservation. Principal coordinate analysis (PCoA) also confirmed the genetic relationships among marigold genotypes (Fig. 4), classified the two species into different clusters, and the results are consistent with UPGMA clustering, supporting the reliability of the genetic groupings.

Conclusion

This study showed that ISSR, EST-SSR, and SSR markers are effective for assessing genetic diversity and population structure in marigold. ISSR markers showed the highest polymorphism and highest resolving power, but SSR and EST-SSR markers also helped distinguish genotypes. These results support the use of multiple molecular marker systems to better understand marigold genetics and guide future germplasm management and variety development.

Declarations

Conflict of interest

The authors declare no competing interests.

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Author Contribution

Conceptualization of research (HD)Designing of the experiments (HD and PVC)Contribution of experimental materials (HD, D V SR, PNK and TNS)Execution of field/lab experiments and data collection (PVCand HD)Analysis of data interpretation and preparation of the manuscript (HD, PVC and PGK)Review and editing of the manuscript (Harish D, PVC and RND).

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Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary materials.

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Figures

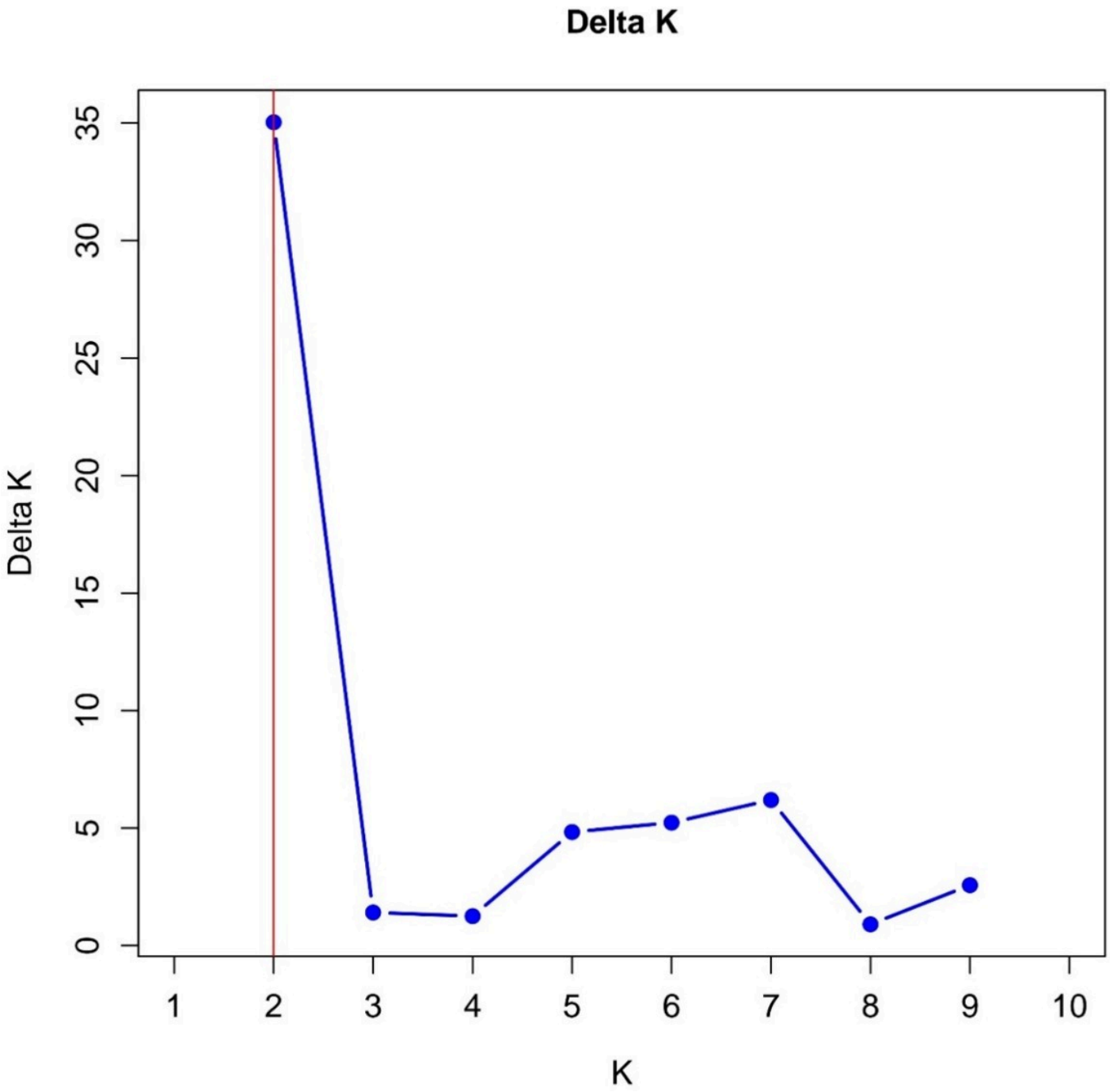

Figure 1

The maximum value of change in ΔK was noted at K= 2; therefore, it is considered an appropriate number for the population as depicted in the graph

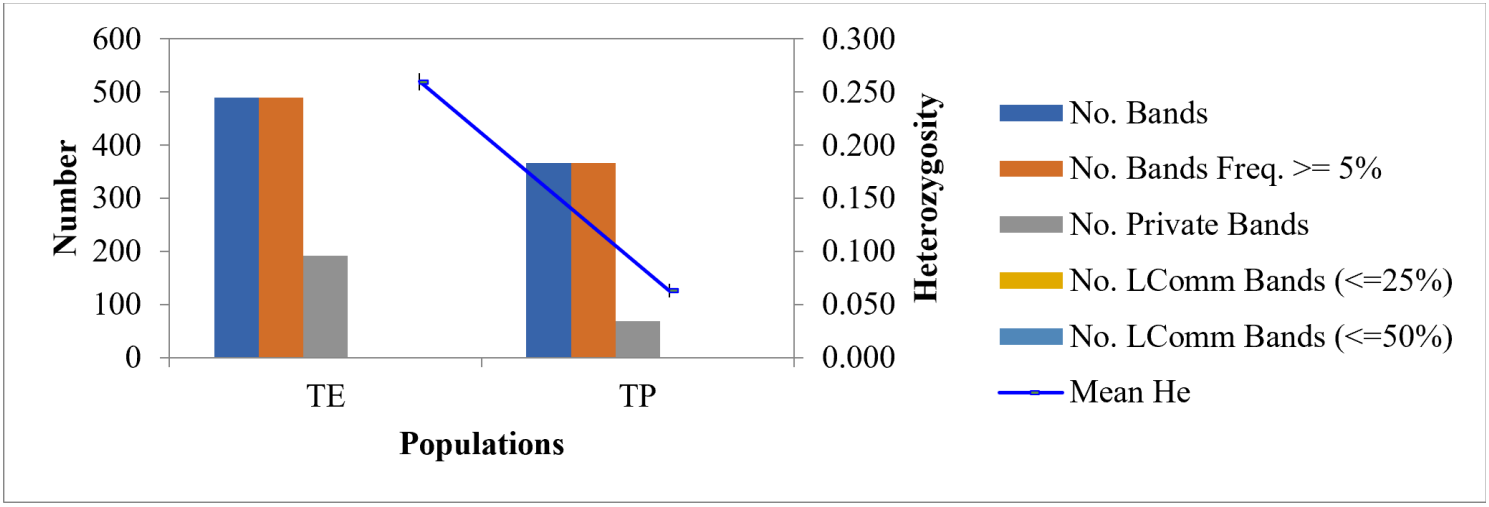

Figure 2

Band patterns across two populations of 19 marigold genotypes

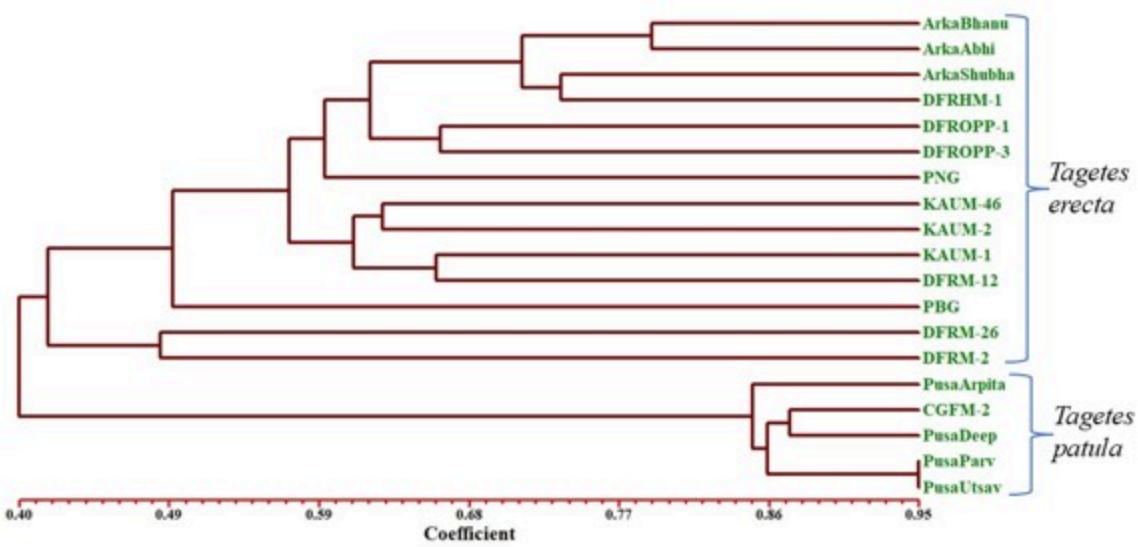

Figure 3

Dendrogram based on hierarchical clustering (UPGMA) using NTSYSpc software. A total of 19 marigold genotypes were grouped in two clusters

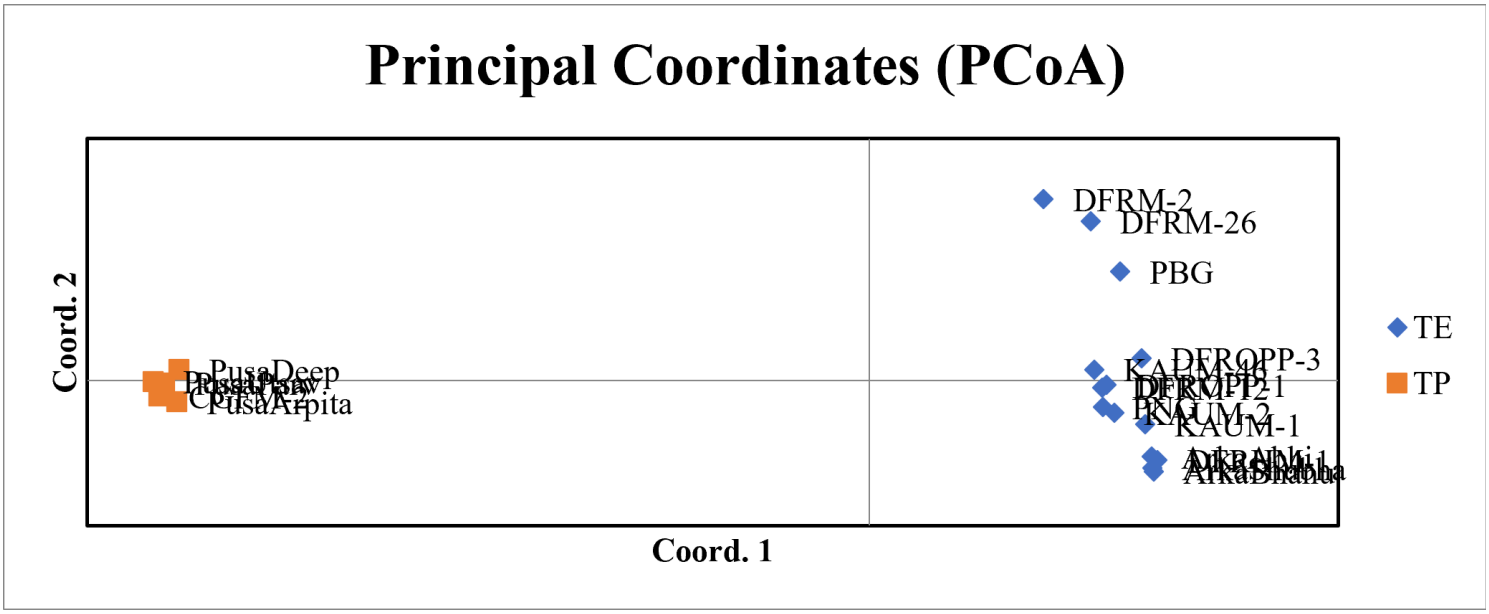

Figure 4

2D PCoA plot drawn using GenAlEx 6.5 software. The two axes represent the first two principal coordinates, which capture the majority of the genetic variation in the data. The genotypes that belong to two different populations were allotted different colors in order to determine the correlation. Genotypes within the same cluster are plotted close to each other, indicating genetic similarity, while genotypes from different clusters are spaced apart, reflecting genetic divergence

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryfileMarigold.docx