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GENETIC DIVERSITY ANALYSIS OF *TAGETES* SPECIES USING PCR BASED MOLECULAR MARKERS

IRUM SHAHZADI, RAZA AHMAD, UMMARA WAHEED AND MOHAMMAD MAROOF SHAH*

Biotechnology Program, Department of Environmental Sciences,
COMSATS Institute of Information Technology, Abbottabad, Pakistan
**Corresponding author's email: mmshah@ciit.net.pk; Ph: 0092-992-383591-6*

Abstract

Tagetes is a genus of medicinally important wild and cultivated plants containing several chemical compounds. Lack of information on variation at molecular level present in *Tagetes* species is paramount to understand the genetic basis of medicinally important compounds. Current study aims at finding genetic variability in *Tagetes* species using random and specific molecular markers. Two primer systems including 25 RAPD and 3 STS (limonene gene) were used to ascertain genetic diversity of 15 *Tagetes* genotypes belonging to different species. We found that 20 of the 25 tested RAPD primers generated stable band patterns with 167 loci of amplification products. The proportion of polymorphic bands was 95.21% for RAPD primers. Three STS primers generated a total of 29 amplification products, of which 96.55% were polymorphic. Homology of genotypes was 53.18% and 51.11% with RAPD and STS primers respectively. The dendrogram obtained revealed that the range of overall genetic distances estimated was 22% to 100% through RAPD and 9% to 100% through STS markers. The findings help to establish that PCR-based assay such as RAPD and STS could be used successfully for estimation of genetic diversity of different genotypes of *Tagetes* that can be used for selection of parents for improvement of the species.

Key words: Genetic diversity, *Tagetes*, RAPD, STS, Monoterpenes, Limonene synthase gene.

Introduction

Tagetes is a genus of flowering marigolds grown under a wide range of climatic conditions.It consists of approximately 216 species divided among 28 genera (Barkley *et al*., 2006). The genus *Tagetes* (marigolds) is represented by 56 species among them 27 are annual and 29 are perennial species (Soule, 1993) throughout the world while three (*T. erecta, T. patula, T. minuta*) are provisionally reported from Pakistan. Marigold plants are stout and branched and can grow up to 60 cm tall. Flowers vary in color from yellow, golden, orange, red to mahogany (Neri *et al*., 2012).

The essential oil of *T. erecta* is reported to have some antimicrobial and antioxidant properties (Gong *et al*. 2012), while the flowers of *T. patula* possess antifungal activity (Romagnoli *et al*., 2005) and on other hand *T. minuta* oil has numerous therapeutic applications (Shahzadi *et al*., 2010b; Xu *et al*., 2012; Shahzadi & Shah, 2015). Biochemical composition of *Tagetes* oil demonstrated various compounds including limonene, ocimene (monoterpenes), dihydrotagetone, tagetone and tagetenone (Kaul *et al*., 2005; Tankeu *et al*., 2013). Monoterpenes are the main constituent and abundant in essential oils of the plant. Though the initial studies pertained to chemical constituents, chemistry and biology of the plant, however, over the last ten years the number of reports on chemotypes and variants of this plant genus have also increased.

Recent advances in molecular biology provided the utility of DNA-based markers. A number of PCR-based techniques can be used for DNA analysis in the field of chemo-variation (Khanuja *et al*., 2005; Akhtar *et al*., 2014). RAPD (Random amplified polymorphic DNA) technique is based on PCR that amplifies random DNA fragments with the use of short single oligonucleotide primers of arbitrary sequence. RAPD method is relatively quick and easy to use, does not require any sequence information and can generate large number of markers which can complement traditional morphological markers (Ntuli *et al*., 2013; Ramireza *et al*., 2014; Shah *et al.,* 2015). RAPD applications reveal polymorphism at DNA level and are efficient tool for investigation of genetic diversity analysis of living organisms (Przyborowski & Sulima, 2010; Rehman *et al*., 2015).

A particular category of molecular or biochemical markers is used for estimating the level of polymorphism and for the estimation of chemodiversity. The medicinal plants contain biochemical compounds and their derivatives that can be used in pharmaceutical industries, perfumery products, cosmetics, flavors and food colorant. There is a need to introduce such plant species to farming community in the country, which, besides meeting the demands of the industry, may help to maintain the standards of quality and biochemical composition. The conventional tools alone are not enough to characterize species which play pivotal role in maintaining, and improving. It was, therefore, imperative to study *Tagetes* species found in Pakistan for their genetic diversity by molecular marker techniques such as RAPD and STS at the initial stage.

Materials and Methods

Plant materials: The seeds and fresh leaf tissues were used to isolate the DNA from 15 genotypes of *Tagetes*. Plant collection is based on the morphologically distinct traits especially flower color and type from plains and hilly areas of Pakistan (Table 1). Seeds of the plants were grown at the research farm of the COMSATS Institute of Information Technology in Abbottabad.

DNA isolation: A modified CTAB DNA extraction method (Doyle and Doyle, 1990; Kim and Hamada, 2005; Shahzadi *et al*., 2010a) was used to obtain high quality DNA with optimum quantity by using seeds and fresh leaf tissues.

Table 1. Sample codes and collection site of *Tagetes* genotypes in Pakistan.

Sample code	Species	Collection site
T1	*T. erecta*	(Sarai Saleh) Haripur
T2	*T. erecta*	(Sarai Saleh) Haripur
T3	*T. erecta*	COMSATS Nursery Abbottabad
T4	*T. erecta*	COMSATS Nursery Abbottabad
T5	*T. patula*	(Sarai Saleh) Haripur
T6	*T. patula*	(Sarai Saleh) Haripur
T7	*T. patula*	COMSATS Nursery Abbottabad
T8	*T. patula*	COMSATS Nursery Abbottabad
T9	*T. patula*	**BZU, Multan*
T10	*T. patula*	COMSATS Nursery Abbottabad
T11	*T. patula*	COMSATS Nursery Abbottabad
T12	*T. erecta*	Kohat
T13	*T. erecta*	Mardan
T14	*T. erecta*	COMSATS Nursery Abbottabad
T15	*T. minuta*	(Balakot) Mansehra

*Baha-ud-dinZakariya University

Randomly amplified polymorphic DNA analysis: Genomic DNA obtained was used as a template for PCR amplification using 25 decanucleotide primers. 20 primers with stable amplification patterns were randomly selected for the study (Table 2). PCR reaction mixture and amplification on thermocycler were the same as described in our previous study (Shahzadi *et al*., 2010a).

STS analysis: Based on the highly conserved amino acid sequence among several plant limonene synthases (*Agastacherugosa*, *Schizonepeta*,*tenuifolia*,*Menthaspicata* and *Perillafrutescens*), degenerate primers were designed by reverse translating the conserved regions using their corresponding genomic codons. Three STS primers with stable amplification products were used for analysis. These primers were 18-nucleotides for each of the forward and reverse primers (Table 3). PCR reaction was carried out in a 20 µL volume. For nested-PCR all ingredients of reaction mixture were same except the template DNA, which was taken from PCR product done already. PCR reactions were carried out in a Perkin Elmer 9700 thermocycler (ABI, Foster City, CA, USA) programmed for an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, primer annealing at 52°C, 54°C, 56°C to 58°C for 1 min and extension at 72°C for 1 min. The reaction was completed with a final run at 72°C for 7 min. Amplified DNA fragments were analyzed by electrophoresis in 0.8% agarose gels in 1xTBE buffer and detected by staining with 10 mg/mL ethidium bromide. Gels were run for 30 min at 100 mA and visualized under UV light.

Data analysis: The results of visible bands of RAPD and STS primers reactions were scored in binary formula with the presence of band scored as unity (1) and its absence scored as zero (0). The degree of homology of examined genotypes and the number of monomorphic and polymorphic amplification products was determined by each primer. Bivariate (1-0) data matrix was used for estimating similarity on the basis of the number of shared amplification products (Nei, 1978). The binary data was used to calculate pair-wise similarity coefficient matrix. The binary matrices were subjected to cluster analysis to generate dendrogram using SAHN command applying unweighted pair group method with arithmetic average (UPGMA) algorithm using NTSYSpc version 2.2 (Rohlf, 2006).

Table 2. Detail information of RAPD primers used in characterizing *Tagetes* genotypes.

Primer	Sequence 5`-3`	Size (nt)	Melting temp. (°C)	Molecular weight (amu)
GL A03	AGTCAGCCAC	10	29.5	2,997
GL A04	AATCGGGCTG	10	29.5	3,088
GL A05	AGGGGTCTTG	10	29.5	3,098
GL A06	CAAACGGGTG	10	29.5	3,004
GL A07	GGGTAACGCC	10	33.6	3,117
GL A09	GTGATCGCAG	10	29.5	3,053
GL A11	CAATCGCCGT	10	29.5	2,988
GL A12	TCGGCGATAG	10	29.5	3,068
GL A13	CAGCACCCAC	10	33.6	2,942
GL A14	TCTGTGCTGG	10	29.5	3,050
GL A15	TTCCGAACCC	10	29.5	2,984
GL A16	AGCCAGCGAA	10	29.5	3,046
GL A17	GACCGCTTGT	10	29.5	3,019
GL A18	AGGTGACCGT	10	29.5	3,068
GL A19	CAAACGTCGG	10	29.5	3,037
GL A20	GTTGCGATCC	10	29.5	3,019
GL B01	GTTTCGCTCC	10	29.5	2,970
GL B02	TGATCCCTGG	10	29.5	3,019
GL B03	CATCCCCCTG	10	33.6	2,924
GL B04	GGACTGGAGT	10	29.5	3,108

Table 3. Gene specific degenerate primer sequences and conserved DNA sequences detail.

Primer	Primer sequence (5′ to 3′)	Length	Product size (bp)	Degeneracy	GC (%)	Tm (ºC)
LSTI-F	CAGCTTGAGTTGATCGAC	18	750	864	50	53.4
LSTI-R	GCCATACACGTCGTAGAT	18		384	50	53.4
LSTII-F	ATCTACGACGTGTATGGC	18	440	384	50	53.4
LSTII-R	AGACTTTGGGACGTCACC	18		1536	56	55.4
LSTIII-F	CTGCAGCTGTATGAAGCT	18	525	1152	50	53.4
LSTIII-R	GCCATACACGTCGTAGAT	18		384	50	53.4

Table 4. Number of amplification products and the degree of homology between the *Tagetes*genotypes for the RAPD primers.

Primer	Number of amplification products			Homology of genotypes (%)
	Total	Monomorphic	Polymorphic	
GL A03	7	1	6	3.80
GL A04	8	0	8	62.85
GL A05	7	0	7	90.47
GL A06	9	0	9	89.52
GL A07	7	1	6	41.90
GL A09	10	1	9	5.71
GL A11	8	0	8	52.38
GL A12	7	0	7	54.28
GL A13	8	1	7	32.28
GL A14	9	0	9	78.09
GL A15	8	1	7	59.04
GL A16	7	1	6	37.14
GL A17	10	0	10	74.28
GL A18	8	0	8	35.23
GL A19	11	0	11	37.14
GL A20	7	1	6	80
GL B01	8	0	8	17.14
GL B02	9	0	9	68.57
GL B03	10	0	10	76.19
GL B04	9	1	8	67.61
Total mean	167	8	159	53.18

Results and Discussions

RAPD analysis: Genetic dissimilarity (estimated as genetic distance) among 15 *Tagetes* genotypes was found by using 20 RAPAD primers for the genetic polymorphism. These primers indicated 167 loci of amplification products with total 757 detectable fragment bands scored against 15 genotypes of *Tagetes* found in northern areas of Pakistan. Of the 167 amplification products 159 (95.21%) were found to be polymorphic and 8 (4.79%) were monomorphic (Table 4). The range of amplification products was 7 to 11 with an average of 8.35 per RAPD primer (Fig. 1). Average homology (53.18%) was observed in 15 genotypes (Table 4).

Table 5 shows average genetic dissimilarity (estimated as genetic distance) among 15 *Tagetes* genotypes using 20 RAPD primers sets. Range of overall genetic distances estimated was from 22% to 100% (Table 5). Maximum genetic distances (100%) were estimated in three comparisons (T3-T13, T6-T14 and T7-T14) while the minimum (22%) genetic distance was observed for one comparison (T9-T10) closely followed by T10-T11 (23%), T13-T14 (25%) and T3-T4 (26%). The remaining genotypes showed insignificant differences.

The dissimilarity coefficient matrix (Nei, 1978) of 15 genotypes of *Tagetes* species based on the data of 20 RAPD primers was used to construct a dendrogram for cluster analysis using UPGMA method (Fig. 2). In general, the dendrogram agreed with the average dissimilarity matrix presented in Table 5. Fifteen genotypes were grouped into four groups (A, B, C and D) in the phenogram. The first group A comprised of 5 (T1, T2, T13, T14 and T12) genotypes of *T. erecta* hybrids, in which T12 out-grouped as it a diverse genotype. T13 and T14 were similar genotypes in this group. Group B consisted of 7 (T5, T9, T10, T11, T6, T8 and T7) genotypes of *T. patula* hybrid. T9 and T10 genotypes were the most similar while T5 and T7 were out-grouped from the rest of this group. Group C comprised 2 (T3 and T4) genotypes of *Tagetes* species. T15 genotype of *T. minuta* was genetically the most distinct from the other genotypes and did not match with any other genotype. 20 RAPD primers were used for the overall genetic diversity study which produced different levels of polymorphism. Overall, genetic distances ranged from 22% to 100% which indicated high level of genetic diversity.

Table 5. Average genetic distances among 15 *Tagetes*genotype using RAPD primers.

Genotype	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15
T1	-														
T2	0.37	-													
T3	0.67	0.42	-												
T4	0.76	0.56	0.26	-											
T5	0.94	0.58	0.84	0.77	-										
T6	0.81	0.50	0.73	0.82	0.39	-									
T7	0.64	0.43	0.78	0.76	0.50	0.33	-								
T8	0.58	0.38	0.67	0.68	0.44	0.29	0.36	-							
T9	0.89	0.61	0.89	0.83	0.32	0.41	0.58	0.31	-						
T10	0.67	0.44	0.67	0.68	0.40	0.38	0.46	0.27	0.22	-					
T11	0.66	0.46	0.70	0.63	0.36	0.42	0.38	0.36	0.29	0.23	-				
T12	0.66	0.58	0.84	0.98	0.67	0.53	0.45	0.65	0.80	0.86	0.76	-			
T13	0.66	0.53	1.00	0.98	0.74	0.58	0.64	0.58	0.56	0.49	0.62	0.54	-		
T14	0.46	0.62	0.83	0.80	0.85	1.00	1.00	0.62	0.60	0.62	0.75	0.78	0.25	-	
T15	0.69	0.53	0.61	0.78	0.73	0.70	0.75	0.69	0.70	0.51	0.69	0.83	0.69	0.82	-

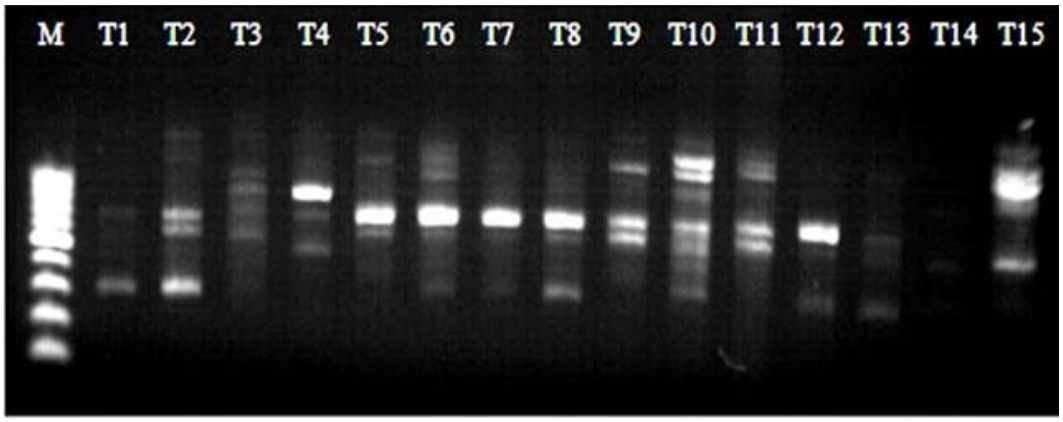

Fig. 1. Ethidium bromide-stained agarose gel showing PCR-amplified products with RAPD primer GL A09. Lanes 1 M: 100-bp DNA ladder; lanes2-16 contains T1 to T15.

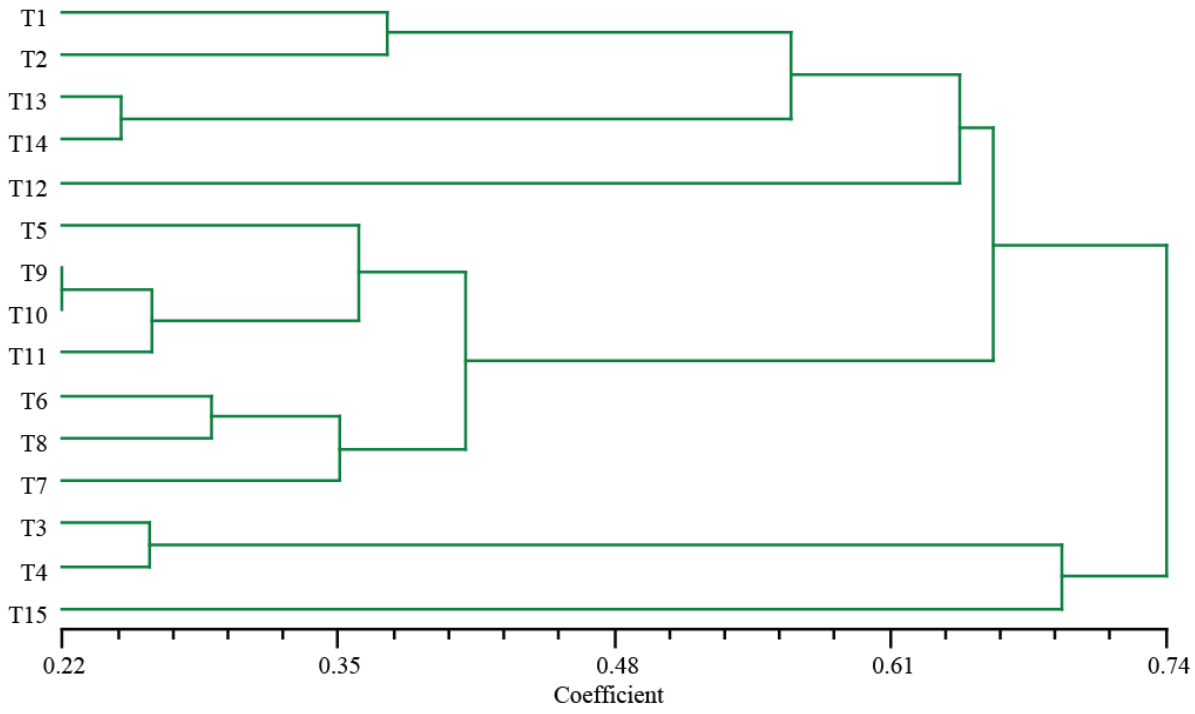

Fig. 2. UPGMA dendrogram of genetic distance based on RAPD between the analyzed genotypes of *Tagetes*.

Table 6. Polymorphism parameters and homology of genotypes using STS primers.

Parameters	Values
Amplification products	29
Polymorphic (%)	96.55
Monomorphic (%)	3.45
Average amplification product per primer	9.67
Homology of genotypes (%)	51.11

STS analysis: We used specific gene primers designed for monoterpenes (Limonene) from *Tagetes* plants species. Three STS primers (LSTI, LSTII, LSTIII) were chosen for the genetic polymorphism and species selection for biochemical compounds (monoterpenes). These primers indicated 29 loci of amplification products with a total of 139 detectable fragment bands scored against 15 genotypes of *Tagetes* found in northern areas of Pakistan. Of the 29 amplified products 28 (96.55%) were found to be polymorphic and 1 (3.45%) was monomorphic (Table 6). The range of amplification products was 7 to 12 with an average of 9.67 per STS primer (Fig. 3). Average homology (51.11%) was observed in 15 genotypes (Table 6).

Genotype T1, T2, T13, and T14 showed non significant amplification products to these STS primers that was also the case in the Nei's genetic diversity findings. UPGMA dendrogram of STS primers illustrated the genetic distance between the studied genotypes (Fig. 4). Table 7 shows average genetic dissimilarity (estimated as genetic distance) among 15 *Tagetes* genotypes against 3 STS primers sets used. Range of overall genetic distances estimated was from 9% to 100%. Maximum genetic distances (100%) were estimated in 3 comparisons (T2-T15, T3-T7, T4-T7 and T4-T9) while the minimum genetic distance (9%) was observed for 1 comparison between T10-T11 closely followed by T3-T4 (10%) and T9-T10 (14%). The remaining genotypes showed insignificant differences.

RAPD is a technique used widely for the analysis of genetic variability and collection of germplasm identification as well as the identification of phylogenetic relationship, cultivars, breeding programs and investigation of lines (Powell *et al*., 1996; Besse *et al*., 2004; Liu *et al*., 2008; Singh *et al*., 2009; Akbar *et al.,* 2011; Sultan *et al.,* 2013). *Tagetes* is a genus of medicinal importance. Several chemical compounds have been isolated but insufficient data is available on molecular analysis of this genus. Generally, molecular marker techniques have been employed in cereal crops such as wheat, maize and rice but less emphasis have been given on medicinally important species like *Tagetes* (Shah *et al*., 2000; Selvi *et al*., 2003; Jun *et al*., 2007; Chen *et al*., 2011; Turi *et al.,* 2012; Yang *et al*., 2014). Genetic diversity analysis of only two *Tagetes* species and their hybrids is reported in very little scientific literature with polymorphism in *T. patula* and *T. erecta* (Pramila *et al*., 2011; Modi *et al*., 2013). It is very timely to ascertain genetic diversity present in the *Tagetes* species to improve this medicinal crop of commercial importance. The increased number of polymorphic amplification enhanced the effectiveness of molecular markers. The polymorphic amplification products in this study confirmed the effectiveness of RAPD markers in establishing the genetic diversity of *Tagetes*, while the use of STS markers is being documented for the first time for studying genetic polymorphism in *Tagetes* genotypes.

The purpose of present study was to identify genetic polymorphism in a set of *Tagetes* genotypes collected from the northern areas of Pakistan. The amplified polymorphic products showed significantly high percentage and degree of genetic diversity in the *Tagetes* species. The present study is a first scientific attempt made in Pakistan to characterize *Tagetes* species on the basis of DNA sequences using RAPD and STS primers. We established PCR-based assay of RAPD and STS that could be used successfully for the estimation of genetic diversity of different genotypes of *Tagetes* species and the reported results may also be contributed to the selection of parents for breeding purposes and mapping population. However, more molecular markers can be used to achieve better understanding about structure and diversity of *Tagetes* species which may lead to the identification of specific markers that could be helpful in tagging of useful genes in crop improvement program.

Table 7. Average genetic distances among 15 *Tagetes*genotype using STS primers.

Genotype	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15
T1	-														
T2	0.00	-													
T3	0.00	0.98	-												
T4	0.00	0.95	0.10	-											
T5	0.00	0.75	0.93	0.90	-										
T6	0.00	0.84	0.84	0.99	0.45	-									
T7	0.00	0.79	1.00	1.00	0.40	0.24	-								
T8	0.00	0.51	0.98	0.94	0.40	0.50	0.20	-							
T9	0.00	0.51	0.98	1.00	0.40	0.65	0.45	0.20	-						
T10	0.00	0.55	0.84	0.99	0.31	0.69	0.50	0.24	0.14	-					
T11	0.00	0.46	0.93	0.90	0.22	0.60	0.40	0.27	0.27	0.09	-				
T12	0.00	0.88	0.88	0.69	0.35	0.73	0.40	0.40	0.69	0.45	0.35	-			
T13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-		
T14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	
T15	0.00	1.00	0.63	0.48	0.68	0.77	0.91	0.73	0.57	0.77	0.86	0.66	0.00	0.00	-

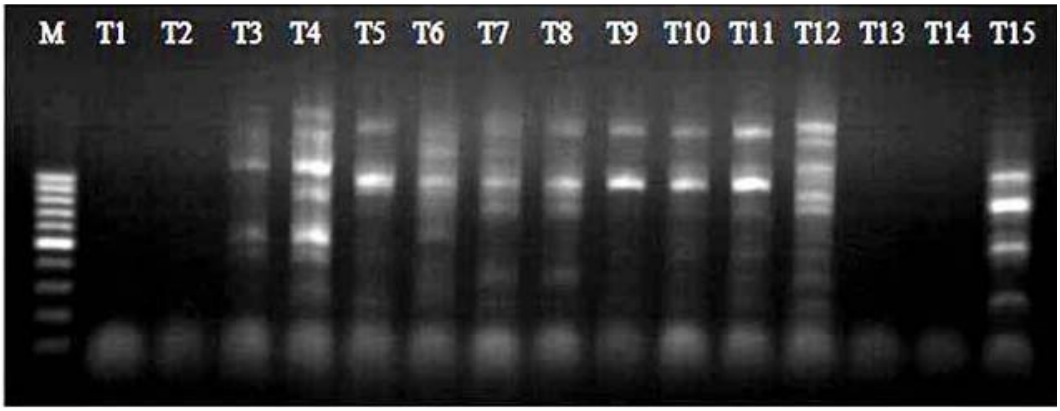

Fig. 3.Ethidium bromide-stained agarose gel showing PCR-amplified products with STS primer LSTIII. Lanes 1 M: 100-bp DNA ladder; lanes2-16 contains T1 to T15.

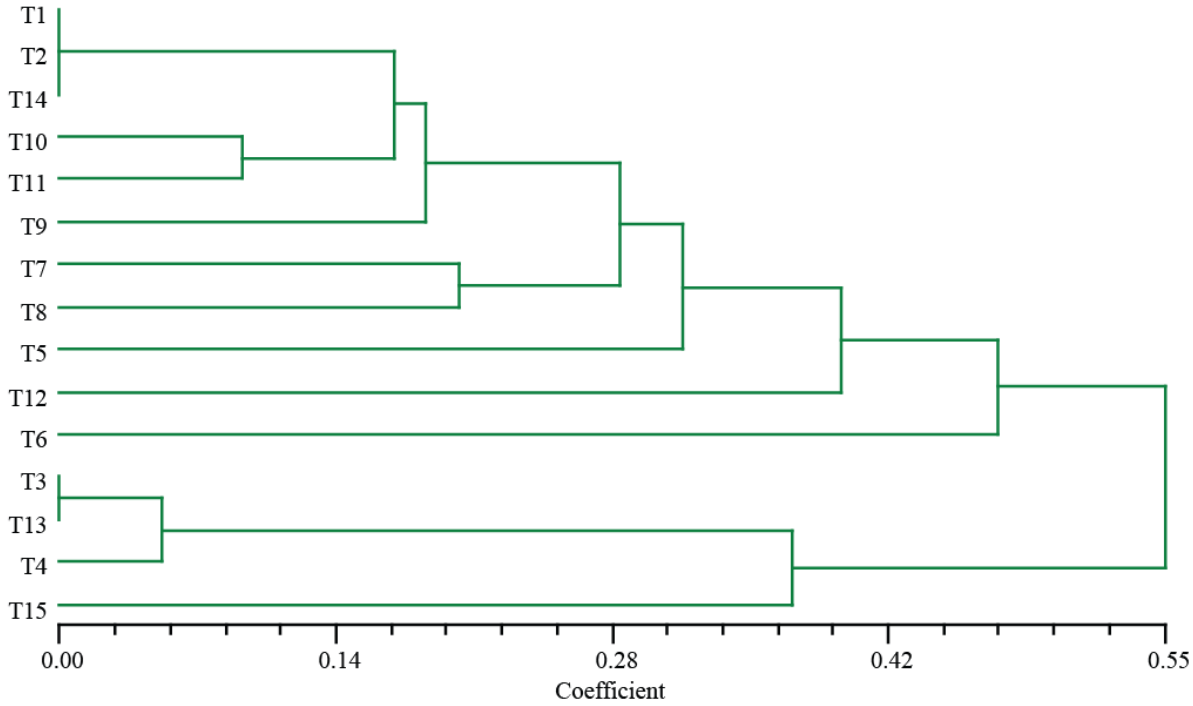

Fig. 4. UPGMA dendrogram of genetic distance based on STS between the analyzed genotypes of *Tagetes*.

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