RESEARCH ARTICLE

Analysis of genetic diversity among male and female pistachio genotypes using start codon targeted (SCoT) makers

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ABSTRACT: Precise investigation of genetic diversity by means of novel molecular tools has made it possible to identify the superior genotypes among various male and female pistachio populations. Cytogenetic studies have shed light on the possible presence of distinct sex chromosomes in male and female genotypes. In this study, 22 start codon targeted (SCoT) primers were used to investigate the genetic diversity of 22 male genotypes and 22 female cultivars of pistachio. A total of 434 loci were produced that 339 loci were polymorphism. The average value of polymorphic information content (PIC), marker index (MI), and resolving power (Rp), ranged from minimum 10, 0.5, and 1, to maximum 31, 11.40, and 17.86% subsequently. The genetic similarity between genotypes, were calculated using Jaccard's coefficient, ranged from 35 to 66%. The cluster analysis divided pistachio genotypes into six groups, and could efficiently differentiate the male and female genotypes. Analysis of molecular variance (AMOVA) classified the total diversity into intra- and inter- population diversities with a high genetic variation (92%) within populations. This study reveals that SCoT marker is a useful and valuable molecular tool to separate male and female pistachios and to determine the genetic diversity among the populations.

KEYWORDS: Cluster analysis, Genotyping, Molecular marker, Molecular variance

INTRODUCTION

Pistachio, one of the major horticultural products in Iran, is of great importance in various economic, social, and environmental terms. Due to Iran's climate, Iranian Pistachio has an exceptional quality, and is of an outstanding superiority to its foreign counterparts. It is indispensable to identify and accurately examine the genetic reservoirs of pistachio for appropriate breeding planning and ensuring the production of the improved cultivars [10]. *Pistacia vera* L. is the most prominent commercial pistachio species, while other species are undomesticated and are considered wild [9, 11]. Domestic pistachio is dioecious, and to bear fruit, both male and female parent plants need to be cultivated. Male and female plants conventionally go under asexual reproduction [20].

The type of pollen influences the time of fruit ripening and the length and percentage of pistachio shells' openness. Pollens from the domestic pistachio species lead to bulkier pistachio nuts and higher percentage of its shells' openness [3].

Domestic pistachio has 2n=30 chromosomes [1]. Pistachio's sexuality is determined using ZW system, and its female genotypes possess two distinct sex chromosomes, and hence they are considered heterogametic [20]. Genetic diversity estimation is one of the fundamental steps in genetic material preservation in gene banks and also in selective breeding programs [6].

DNA markers indicate individual variations on DNA level and have high polymorphism, random genomic

distribution, and high frequency. They are neutral phenotypically, unresponsive to environment variations, non-dependent of growth stage, and measurable in any in vitro tests. Nowadays, a wide range of DNA markers are available, and criteria including their genomic locus and relative distribution in genome (in cases of markers with known genetic locus) need to be considered when selecting one [12]. Recently, several SNP markers for sex determination of pistachios have been introduced as well [10].

Start Codon Targeted (SCoT) marker is a novel marker based on Polymerase Chain Reaction (PCR) which is designed based on the short conserved sequences in plant genes surrounding the ATG initiation codon. Like RAPD and ISSR markers, this marker is dominant. Each of its primers acts as both forward and reverse primers. Moreover, the primary information regarding the genome is not required when designing the primers for this marker. Yet it is more reproducible in comparison with RAPD and ISSR [2]. It is assumed that the length and the annealing temperature of these primers are not the only factors determining the reproducibility of band pattern and also magnesium chloride concentration is important [2, 16]. This method's main advantages include its low cost, ease and speed of implementation, high polymorphism, extensive genetic data exposure, and widespread primers throughout the plants' genome. SCoT marker is used in genetic diversity research, genetic analysis, gene mapping of Quantitative Trait Loci (QTLs), bulk screening method, and DNA fingerprinting [2]. Guo et al. [7] declared that genetic analysis using SCoT marker is a simple, efficient, and reliable method for detection and preservation of plant germplasms and their relative species.

DNA finger printing of 24 tetraploid potato cultivars and its population segregation with three molecular markers of SCoT, ISSR, and RAPD showed that SCoT marker resulted in higher polymorphic information content, diversity index, resolving power, and marker index in comparison with other markers [13]. Molecular markers SCoT and IRAP were used to evaluate 50 wild pistachio species collected from different geographic regions of Iran, and SCoT marker produced high polymorphism in wild pistachio species assessment. The results of this study were announced to be helpful in germplasm management and natural resources preservation and breeding methods improvements [21]. Using SSR marker, genetic diversity of Iranian and non-Iranian cultivars of pistachio and other species of *Pistacia* were

assessed, and Iranian and non-Iranian species were separated with great significance [14]. ISSR Marker was also able to segregate male and female genotypes of a total 56 pistachio genotypes, and high polymorphism in female cultivars, and higher intra-population diversity relative to inter-population diversity were observed [5].

This study was conducted based on SCoT marker capability in plants genetic diversity evaluation and presence of distinct sex chromosomes in order to investigate genetic diversity among male and female phenotypes of pistachio.

MATERIALS AND METHODS

Plants samples

Fresh leaves of 22 male genotypes and 22 female cultivars available at Pistachio Research Center (Rafsanjan) were collected. The list of female cultivars and male genotypes is presented in Table 1.

Table 1. Pistachio cultivars and genotypes used in this study

Code	Genotype*	Sex	Code	Cultivar	Sex
M1	K34	M	F1	Javadaghayee	F
M2	K37	M	F2	Badaminishkalaghi	F
M3	N1	M	F3	Vahedi	F
M4	K38	M	F4	Shasti	F
M5	K41	M	F5	Beheshtabadi	F
M6	R25	M	F6	Momtaztagabadi	F
M7	R27	M	F7	Sirizi	F
M8	K33	M	F8	Phandoghireez	F
M9	K39	M	F9	Jandaghi	F
M10	K40	M	F10	Harati	F
M11	R26	M	F11	Kalehghoochi	F
M12	R24	M	F12	Khanjaridamaghani	F
M13	R23	M	F13	Ghafoori	F
M14	N4	M	F14	Lacksiri	F
M15	N12	M	F15	Seifadini	F
M16	N10	M	F16	Bayaz	F
M17	N5	M	F17	Sephidpestehnoogh	F
M18	R19	M	F18	Italiaee	F
M19	R20	M	F19	Badamiravar	F
M20	N6	M	F20	Badamizerand	F
M21	K42	M	F21	Ahmadaghaee	F
M22	R21	M	F22	Akbari	F

*Male genotypes are labeled by their origin, with K: Kerman, R: Rafsanjan, and N: Nasiriyah of Kerman. M: male, F: female

Table2. List and sequence of SCoT primers, Number of bands, the number of polymorphic bands, polymorphism Percent, Polymorphism information content (PIC), Marker Index (MI) and Resolution Power (Rp) for used SCoT primers in this study.

Code	Sequences	Ta °(C)	No. of bands	No. of polymorphic bands	polymorphism(%)	(PIC)	(MI)	(Rp)
S2	CAACAATGGCTACCACCC	54	16	12	75	0.12	1.46	2.77
S3	CAACAATGGCTACCACCG	54	15	14	93	0.26	3.67	5.59
S6	CAACAATGGCTACCACGC	54	23	21	91	0.25	5.44	8.86
S7	CAACAATGGCTACCACGG	54	27	27	100	0.27	7.42	10.5
S8	CAACAATGGCTACCACGT	52	15	14	93	0.18	2.62	3.63
S12	ACGACATGGCGACCAACG	54	13	12	92	0.24	2.90	4.90
S13	ACGACATGGCGACCATCG	54	37	36	97	0.31	11.40	17.86
S16	ACCATGGCTACCACCGAC	54	22	18	81	0.20	3.64	6.5
S17	ACCATGGCTACCACCGAG	54	26	25	96	0.26	6.65	9.40
S18	ACCATGGCTACCACCGCC	54	15	12	80	0.25	3.01	5.95
S21	ACGACATGGCGACCCACA	54	24	22	91	0.21	4.27	7.31
S24	CACCATGGCTACCACCAT	54	20	17	85	0.21	3.73	6.18
S25	ACCATGGCTACCACCGGG	56	31	31	100	0.24	7.46	10.72
S27	ACCATGGCTACCACCGTG	54	8	7	87	0.21	1.50	2.54
S28	CCATGGCTACCACCGCCA	58	4	1	25	0.12	0.5	1
S29	CCATGGCTACCACCGGCC	58	26	25	96	0.28	7.13	10.86
S30	CCATGGCTACCACCGGCG	58	27	27	100	0.17	4.75	6.04
S31	CCATGGCTACCACCGCCT	58	18	15	83	0.10	1.57	2.09
S33	CCATGGCTACCACCGCAG	56	16	16	100	0.23	3.76	4.77
S34	ACCATGGCTACCACCGCA	54	22	20	90	0.25	5.01	7.81
S35	CATGGCTACCACCGGCCC	61	18	18	100	0.25	4.66	6.72
S36	GCAACAATGGCTACCACC	54	11	9	81	0.17	1.56	2.31
Total			434	399				

DNA Extraction

Using CTAB method with slight modifications, genomic DNA was extracted from leaf samples stored at minus 80 degrees of Celsius [4]. DNA quality and quantity was measured using ultraviolet spectrophotometer at 260 nm and 280 nm wave length and agarose gel electrophoresis.

PCR and Electrophoresis of Amplified Products

Amplification reaction of each sample was held in a total volume of 20 microliters, containing 50 nanograms of DNA, and was conducted with 22 SCoT primers separately (Table 2). The amplification stages included initial denaturation at 94 ˚C for 3 minutes, 40 cycles of denaturation at 94 ˚C for 30 seconds, primer annealing for 30 seconds and at each primer's optimum temperature (Table 2), extension at 72 ˚C for 2 minutes, and the final extension at 72 ˚C for 5 minutes were all conducted in Thermal Cycler Bio-Rad, C1000tm. Amplified products were loaded into the wells of a 1.5%

agarose gel in 0.5X TBE buffer, and electrophoresis was conducted at voltage of 120 for two hours. Then, gel imaging was performed using Gel Doc equipment.

Data Analysis

PCR-amplified fragments were scored either one (1) or zero (0) based on the presence or absence of a band, respectively. The similarity matrix was calculated using Jaccard's similarity coefficient and cluster analysis was performed based on complete linkage method in NTSYS pc 2.02e software. For each primer marker index (MI) [17], polymorphic information content (PIC) [23] and resolving power (Rp) [18] were calculated separately. Genetic diversity assessment of male and female pistachio populations and Principal Components Analysis (PCOA) were done using GenAlEx software, and analysis of molecular variance (AMOVA) was applied to determine the intra- and inter- population diversities of male and female pistachios [15].

RESULTS and DISCUSSION

The 22 SCoT primers generated a total of 434 amplified loci form 44 genotypes. The highest and the lowest amplified loci belonged to S13 primer with 37 amplified loci, and S28 with 4 amplified loci, respectively (Table 2). Each primer generated 20 loci on average. 399 of 434 amplified loci displayed polymorphism (92%), and S13 primer generated the most number of polymorphic loci (36) (Figure 1). The average number of polymorphic loci attributed to each primer was equal to 18. Farzad et al. [5] reported a total of 178 amplified loci using 12 ISSR primers on 56 pistachio genotypes, and 169 of the loci displayed polymorphism. The disparity observed between the result of the present study and the Farzad et al. [5] might be due to the difference in the number of genotypes investigated and the types of markers used in each study. Based on the results of two studies utilizing ISSR markers on pistachio samples, ISSR markers generated less amplified loci and also less polymorphic loci relative to SCoT markers, and it seems that SCoT markers are more powerfull in generating polymorphism in pistachio genotypes [5, 22].

The average of PIC varied from 10% (lowest value) relating to S31 primer up to 31% (highest value) regarding S13 primer. S13 primer demonstrated the highest MI and Rp values among all primers (Table 2). In a study on 10 tomato samples exploiting 10 SCoT and 10 ISSR markers, SCoT markers exhibited superior results in every aspect including percentage of polymorphism, polymorphic information content, resolvign power, and marker index compared to ISSR markers [19].

According to the results of similarity matrix, the highest genetic similarity observed between K37 and K38 genotypes (69% similarity), and the least genetic similarity was found between the Italiaee cultivar and N5 genotype (35% similarity). Since K38 and K37 genotypes share common origin (Kerman), their significant level of genetic similarity seems rational. Among male genotypes, R23 had the lowest genetic similarity average (45%) with female cultivars. In terms of augmenting genetic diversity, this genotype might proven to be superior to other male genotypes as a male partner for female cultivars of this study in breeding programs (taking the flowering synchrony into account). Using similarity matrix results, genetic similarity of all female cultivars with male genotypes were determined. Based on this study, Javadaghayee, Shasti, Beheshtabadi, Sirizi, Jandaghi, Harati, Khanjaridamaghani, Lacksiri, Seifadini, Bayaz, Sephidpestehnoogh, Badamiravar, Badamizerand, and Akbari cultivars held the highest genetic distance from R23 male genotype. Badaminishkalaghi, Vahedi, Phandoghireez, Kalehghoochi, Ghafoori, and
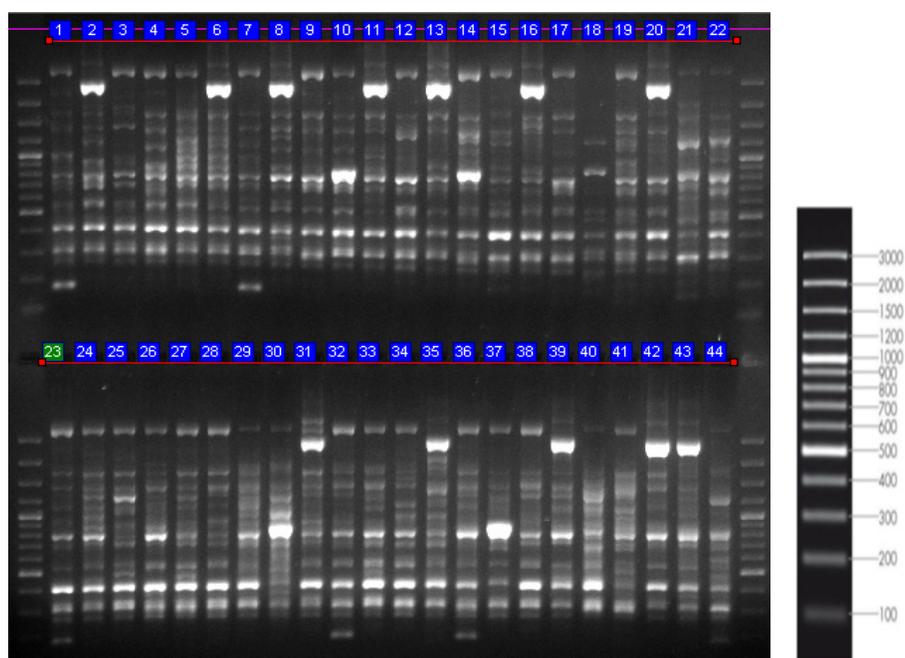
Figure 1. Electrophoresis pattern of amplified fragments of 44 pistachio genotypes by primer S13 on agarose gel (1.5%)

Ahmadaghaee cultivars owned the highest genetic distance from R27 male genotype, and Momtaztagabadi possessed the highest genetic distance from R25 male genotype. Due to pistachio's high heterogeneity, crossing genotypes with the least genetic similarity can be effective in increasing genetic diversity. Therefore, in order to generate more genetic diversity, male genotypes with a high genetic distance from female cultivars, are more favorable for cross-fertilization with a given female cultivar .

According to the dendrogram (Figure 2), the cluster analysis of the investigated genotypes divided genotypes into 6 main groups and 1 single-genotype group in 0.48 genetic similarity. The first group consisted of three female cultivars, Javadaghayee, Shasti and Beheshtabadi; Shasti and Beheshtabadi cultivars were also in the same group in the aforementioned ISSR marker-based study [5].

The second group included 6 male genotypes: R19, K37, K38, K39, K42, and N5. The third group was made up of 14 female and 3 male genotypes; Momtaztagabadi, Ghafoori, Phandoghireez, Kalehghoochi, Sirizi, Jandaghi, Khanjaridamaghani, Harati, Sephidpestehnoogh, Seifadini, Lacksiri, Badamiravar, Bayaz, Badamizerand, N6, K34, and R20. Based on Taghizad et al. [22] classification, Seifadini, Kalehghoochi, and Phandoghireez were categorized in one group, however, in contrast to this study,
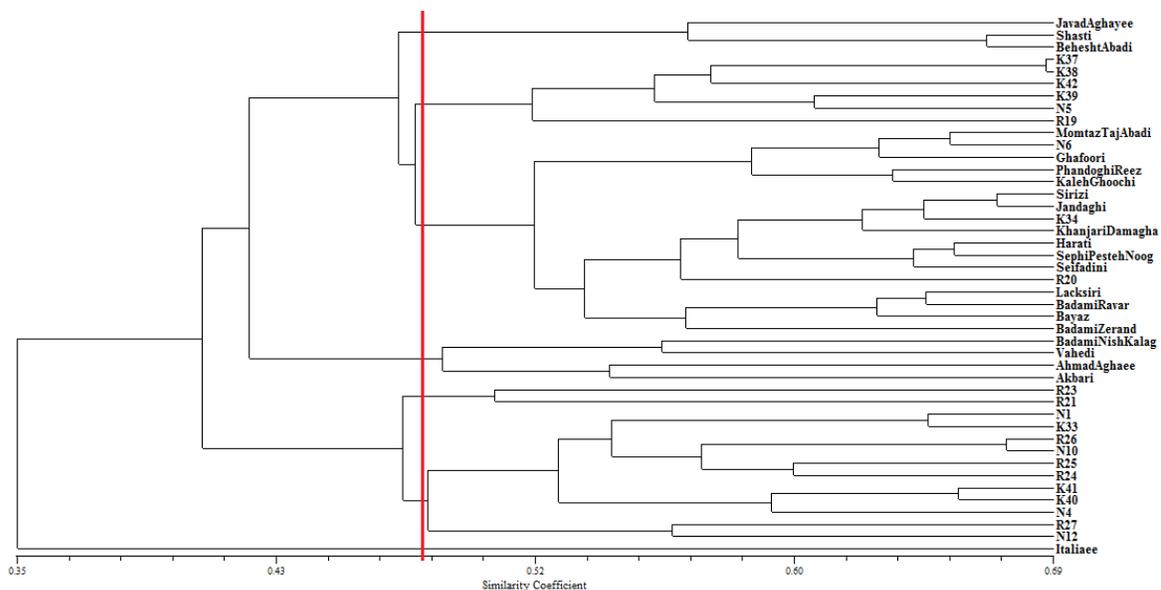
Khanjaridamaghani and Sephidpestehnoogh cultivars did not belong to the same category in their study. As both Badamiravar and Badamizerand belong to Almonds Family, their existence in the same group, and in turn their genetic similarity is justifiable.

The fourth group included four female cultivars, Badaminishkalaghi, Vahedi, Ahmadaghaee, and Akbari, which are closely related in terms of their fruit shape. Corresponding to the previous classifications, Ahmadaghaee and Akbari were in the same group [5, 22].

Two male genotypes R21 and R23 which were collected from Ravar, lied in the fifth group. In the sixth group, SCoT marker was thoroughly able to distinguish male and female genotypes. Resembling the second group, this group also contained solely eleven male genotypes; N1, K33, R26, N10, R25, R24, K41, K40, N4, R27, and N12. Eventhough the aforementioned genotypes vary regarding their origin (Nasiriyah, Kerman, Rafsanjan), they probably share the same parents, which are present in the same group. In the research done by Farzad et al. [5] male genotypes R27, K33, K40, R25, R24, and K41 were classified in the same group as well.

Being a foreign genotype, Italiaee cultivar was categorized individually and distinguished from other categories. This could be due to its high genetic distance from other genotypes of this study.

PCoA was also conducted to investigate the genetic

Figure 2. Dendrogram of 44 genotypes and cultivars of male and female pistachio based on 22 SCoT primers using Complete linkage method.

Figure 3. Biplot two-dimensional graph based on the first and two components (PC1 and PC2) to study the genetic diversity of studied male and female pistachio populations.

diversity of male and female populations (Figure 3). The characteristic value of all components was calculated, but the results were interpreted based on the first two components (first component (27.5) and second component (18.97)), which justify great percentages of variations. Based on bi-plot analysis, male and female genotypes of pistachio were observed in four regions in bi-plot area. Positive regions of both first and second components included only female cultivars. In the region where the first component is positive and the second one is negative, a mixture of male genotypes and female cultivars was witnessed. In the region where both components are negative, only male genotypes existed, and in the region of negative first component and positive second component, a combination of both male genotype and female cultivars was observed. The accumulation of genotypes in one region of the graph indicated the genetic resemblance of those genotypes. In complete congruence with this study, in the research conducted by Farzad et al. [5] using ISSR markers, Harati, Shasti, and Beheshtabadi cultivars were found in one region all together, and Ghafoori, Khanjaridamaghani, Bayaz, Lacksiri, and Badamizerand were observed in one region as well. However, in plain contrast to their research results, the above cultivars alongside Ahmadaghaee and Akbari cultivars were observed in a separate region. This discrepancy can stem from the distinct markers used in each of the two studies.

Analysis of molecular variance revealed that 53.87 is related to the variance of the total population, and 8% (4.22) corresponds to inter-population variance, and 92% (49.65) correlates with intra-population variance (Table 3). According to a research conducted on 36 female cultivars and 20 male genotypes of pistachio by means of ISSR markers, it was revealed that the contribution of intra-population diversity is more significant than that of inter-population diversity [5], as found out in this study. High level of intra-population variance manifests the prominent genetic diversity among genotypes in each population of the experiment, which can be attributed to the impact of the male parent and genetic recombination in mentioned genotypes.

Based on this experiment's results, loci polymorphism levels in female and male populations were 77.19 and 78.11 percent, respectively. In contradiction to the results of this analysis, Farzad et al. [5] proved the polymorphism to be higher in female cultivars than in

Table 3. Analysis of molecular variance using SCoT markers between and within male and female Pistachio populations.

Sources of variance	df	Est. Var.	% Var.
between populations	1	4.22	8%
within populations	42	49.65	92%
Total	43	53.87	100%
PhiPT		0.078 (p=0.01)	

male genotypes. This difference can be attributed to the predominance of female cultivars to male genotypes in terms of number in the prior study. In an experiment on 16 male genotypes and 8 female cultivars of pistachio using RAPD markers, male genotypes displayed higher polymorphism relative to female cultivars [8]. Hence, it can be concluded that the number of genotypes in each population of study, has a significant role in determining the polymorphism in the population.

CONCLUSION

Following juxtaposition of the results of this study and other studies on pistachio genotypes, it was found that SCoT marker generated more amplified fragments than ISSR marker, and it appeared more successful in producing polymorphism. This confirms the superiority of SCoT marker over ISSR marker in forming polymorphism. SCoT marker was also able to completely distinguish male and female genotypes, and it was observed that male R23 genotype had the least genetic similarity with the investigated cultivars. Due to pistachio's high heterogeneity, crosses of genotypes with the lowest genetic similarity can be effective in elevating genetic diversity. Thus, male genotypes with a high genetic distance from female cultivars seem suitable for cross- fertilization with cognate female cultivars, particularly with the aim of creating more genetic diversity.

In this research, it was found that SCoT markers have high values of marker index, resolving power, and polymorphic information content, and can be recommended as a promising tool in genotyping. Furthermore, S13 primer proved to be an appropriate candidate in pistachio fingerprinting experiments, Owing to its higher MI and Rp in comparison to other studied primers.

ACKNOWLEDGMENTS

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ارزیابی تنوع ژنتیکی در میان ژنوتیپ‌های نر و ماده پسته با استفاده از نشانگر SCoT

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چکیده

بررسی دقیق تنوع ژنتیکی با استفاده از ابزارهای مولکولی نوین، امکان شناخت ژنوتیپ‌های برتر جمعیت‌های مختلف نر و ماده پسته را فراهم کرده است. بررسی‌های سیتوژنتیکی نشان داده است که در پسته احتمالا کروموزم‌های جنسی متفاوتی در ژنوتیپ‌های نر و ماده وجود دارد. در این تحقیق، به‌منظور بررسی تنوع ژنتیکی ۲۲ ژنوتیپ نر و ۲۲ رقم ماده پسته از ۲۲ آغازگر SCoT استفاده شد. در مجموع ۴۳۴ مکان تکثیر شده شامل ۳۳۹ مکان چندشکل به دست آمد. میانگین محتوای چندشکل، شاخص نشانگری و قدرت تفکیک بر پایه داده‌های SCoT، به ترتیب از حداقل ۱۰ درصد، ۰/۵ و ۱ تا حداکثر ۳۱ درصد، ۱۱/۴۰ و ۱۷/۸۶ متغیر بود. با استفاده از ضریب تشابه جاکارد حداکثر شباهت ژنتیکی بین ژنوتیپ‌ها ۶۹٪ و حداقل شباهت ژنتیکی ۳۵٪ بدست آمد. تجزیه خوشه‌ای ژنوتیپ‌های پسته را در شش گروه در سطح ۴۸ درصد شباهت تقسیم نمود و به خوبی توانست ژنوتیپ‌های نر و ماده را از هم تفکیک کند. برای بررسی دقیق‌تر تنوع ژنتیکی، از ماتریس تشابه و تجزیه به مولفه‌های اصلی نیز استفاده شد. آنالیز واریانس مولکولی تنوع کلی را به دو تنوع بین و درون جمعیت‌ها تقسیم نمود. که سطح بیش‌تر تنوع، به درون جمعیت‌ها (۹۲٪) تعلق داشت. نتایج این تحقیق نشان داد که، نشانگر SCoT ابزار مولکولی مفید و ارزشمند برای تفکیک جمعیت‌های نر و ماده پسته و تعیین تنوع ژنتیکی بین جمعیت‌های فوق می‌باشد.

کلمات کلیدی: تجزیه خوشه ای، ژنوتیپ، نشانگر مولکولی، واریانس مولکولی