

## Evaluation of Genetic Diversity of Twenty-eight Sweet Cherry Genotypes by Morphological Traits and SCoT Markers in the Northwest of Iran

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### ABSTRACT

Sweet cherry (*Prunus avium* L.) is mainly grown in temperate climate countries. Because of the economic importance of sweet cherry and its high genetic diversity in Arasbaran (a region in northwestern Iran), the selection of superior genotypes as resistant rootstocks has great importance for breeding programs. In this study, the genetic diversity of 28 genotypes of sweet cherry was by 11 qualitative and nine quantitative traits and SCoT markers, in the Ahar Afil area (a region of Arasbaran). In the first experiment, quantitative and qualitative characteristics of the fruits, leaves, trees as well as flowers were evaluated. Results of simple correlation coefficients showed a positive and significant correlation among some of the measured morphological traits. Cluster analyses of the morphological traits classified the genotypes into two main groups. In the second experiment, SCoT markers, by application of 12 primers, were used for genetic diversity analysis of the sweet cherry genotypes. The results, 89.9% of polymorphism was detected by 12 primers and the number of polymorphic bands per primer was between 2 to 9 bands, with an average of 5.3. The number of observed ( $N_a$ ) and effective ( $N_e$ ) alleles, Marker index (MI), resolving power (RP), and an index for primer was an average of 11, 8.27, 3.78, and 5.07, respectively. A high variation was observed in the grouping of genotypes through cluster analysis. Cluster analysis based on Dice similarity coefficient matrix and structure analysis classified the populations into eight main groups. Our results showed a high level of genetic diversity in 28 sweet cherry genotypes. This study will be helpful for the conservation and management of sweet cherry genetic resources for further breeding programs.

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### Introduction

Sweet cherry is one of the world's important and attractive fruits (Naderiboldaji *et al.*, 2008), of the genus *Prunus*, family Rosaceae, mainly grown in temperate climate countries (Kazantzis *et al.*, 2011). According to the FAO (2020), Turkey is the world's largest cherry producer followed by the USA. Sweet cherry is one of the most important fruit crops in Iran and this country is one of the most productive countries in the world, with about 28000 hectares of orchards (Mozafari *et al.*, 2011).

There is a high morphological and genetic variability in sweet cherry populations exist in various parts of Iran. Because of the economic importance of sweet cherry in Arasbaran, genetic diversity study in this region required, to select superior genotypes as resistant rootstocks for breeding programs. Moreover, morphological and genetic diversity researches on the sweet cherry in Ahar city has restricted in comparison with the other region of Iran.

Estimation of genetic diversity in sweet cherry is evaluated by several technique, including



morphoagronomical characteristics (Rodrigues *et al.*, 2008) and various molecular markers, such as random amplified polymorphic DNA (RAPD) (Lisek *et al.*, 2006; Colagar *et al.*, 2013), RAPD and simple sequence repeats (SSR) or microsatellites (Stanys *et al.*, 2012; Colagar *et al.*, 2013), amplified fragment length polymorphism (AFLP) (Struss *et al.*, 2001), inter-simple sequence repeat (ISSR) (Chengxiang *et al.*, 2011) and sequence-related amplified polymorphism (SRAP) (Abadian *et al.*, 2012).

In recent years, among the many types of molecular markers, start codon targeted (SCoT) polymorphisms has been demonstrated to be a new alternative and capable marker techniques for population genetic studies. These markers are reproducible and short conserved region flanking the ATG start codon in plant genes (Collard *et al.*, 2009), and use a single 18-mer primer in the polymerase chain reaction (PCR) assays and higher annealing temperature (50°C), standard agarose gel electrophoresis is used to resolved amplicons (Zhang *et al.*, 2015). The genetic diversity and structure of different species have been successfully evaluated by SCoT markers, including Grape (Guo *et al.*, 2012), Tunisian Citrus species (Mahjbi *et al.*, 2015), Summer Squash (Xanthopoulou *et al.*, 2015), Durum Wheat (Etminan *et al.*, 2016), *Physalis* (Feng *et*

*al.*, 2018) and *Pistachio* (Malekzadeh *et al.*, 2018).

This, the aims of the present study were the analysis of quantitative and qualitative unique morphologic characteristics of some sweet cherry (*prunus avium* L.) genotypes in the Ahar zone and also the investigation of the genetic diversity of sweet cherry trees in this region by SCoT markers.

## Materials and Methods

### Plant materials

Twenty-eight cultivated trees of sweet cherry genotypes (A1, A2, A3, and A28) were selected from Ahar region in the northwest of Iran. The Ahar region located Latitude: 38.4894° N, Longitude: 47.0684° E and the elevation of this region is 1376 meters above sea level. These genotypes were chosen for morphological and pomological studies based on important quantitative and qualitative traits as well as molecular genetic diversity. The genotypes were collected in same growing conditions.

### Morphological analyses

The evaluated characteristics, units and measuring scales are shown in table 1. The morphological traits evaluated based on UPOV descriptors (UPOV, 2006) with nine replications.

**Table 1.** Quantitative and qualitative traits and their units of measurement.

No.	The studied traits	Measuring methods
1	Tree vigour	weak- very strong (1-9)
2	Flowering time	Too early- too late (1-9)
3	Fruit Tissue	Soft- very firmness (1-7)
4	Skin color	Yellow to black like (1-8)
5	Flesh color	Cream-Dark red (1-5)
6	Fruit size	Very small- Very large (1-5)
7	Titrateable acidity	little- much (1-3)
8	PH values	little- much (1-3)
9	Fruit ripening time	Too early- too late (1-9)
10	Flesh/ Stone	little- much (1-3)
11	Fruit weight	Digital scale (g)
12	Flesh weight	Digital scale (g)
13	pit weight	Digital scale (g)
14	Fruit width	Caliper (mm)
15	Fruit length	Caliper (mm)
16	Fruit shape	Heart shaped, renal shape (1,2)
17	Leaf length	Caliper (mm)
18	Leaf Width	Caliper (mm)
19	Leaf area	leaf-area meter (cm <sup>2</sup> )
20	Fruit tails length	Caliper (mm)

## DNA extraction and PCR procedure

Genomic DNA was extracted from 100 mg of fresh leaves by the modified CTAB extraction method (Doyle and Doyle, 1987). The quality

and yield of DNA samples were examined on the agarose gel and the 260 to 280 nm ratio by spectrophotometer. Twelve SCoT markers were selected according to prior studies (Table 4).

**Table 4.** Some information on amplified fragments and genetic parameters for 12 SCoT primers used in this study.

Primer	Primer sequence (5'→3')	Annealing Temperature (C)	Total Fragments	Polymorphic bands	%Polymorphic	PIC	EMR	MI	RP	Na	Ne
SCoT 1	CCATGGCTACCACCGCAC	60	6	6	100	0.78	6	4.68	4.64	12	8.85
SCoT 2	ACCATGGCTACCACCGCA	58	3	2	66	0.56	1.3	0.73	3.28	5	3.82
SCoT 3	CCATGGCTACCACCGCAG	60	4	3	75	0.71	2.25	1.60	5.07	7	6.09
SCoT 4	GCAACAATGGCTACCACC	56	6	6	100	0.77	6	4.62	5.5	12	9.68
SCoT 5	CAACAATGGCTACCACGG	56	6	6	100	0.53	6	3.18	2.64	12	7.59
SCoT 6	CAACAATGGTACCACGG	56	8	8	100	<b>0.81</b>	<b>8</b>	<b>6.48</b>	4.46	<b>16</b>	<b>11</b>
SCoT 7	ACGACATGGCGACCATCG	58	9	9	100	<b>0.86</b>	<b>9</b>	<b>7.74</b>	5.21	<b>18</b>	<b>12.38</b>
SCoT 8	ACCATGGCTACCACCGCC	60	5	5	100	0.75	5	3.75	<b>8.07</b>	10	8.16
SCoT 9	ACCATGGCTACCACCGCA	58	4	3	75	0.64	2.25	1.44	3.85	7	5.21
SCoT 10	ACGACATGGCGACCCACA	58	5	4	80	0.70	3.2	2.24	4.78	9	6.83
SCoT 11	ACGACATGGCGACACCG	60	7	7	100	<b>0.81</b>	7	5.67	5.92	14	10.9
SCoT 12	CAACAATGGCTACCACCG	56	6	5	83	0.78	4.16	3.24	<b>7.42</b>	11	8.77
Mean	-	-	5.75	5.3	89.9	0.72	5.01	3.78	5.07	11	8.27

(PIC) polymorphic information content; (MI) marker index; (RP) primer resolving power; (Na) observed number of alleles; (Ne) effective number of alleles (Kimura and Crow, 1964).

For PCR reaction, each amplification reaction contained 7.5 µl master mix (Ampliqon, Denmark), 1.2 µM primers, and 20 ng genomic DNA and dH<sub>2</sub>O up to 12 µl final volume. PCR amplification program was applied as follows: pre-denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing, the reaction is incubated at 56-60°C for 45 s and primer elongation at 72°C for 45 s. The final extension was 5 min at 72°C. The amplification reaction products were detected by electrophoresis on 1.5% agarose gels. The DNA fragments was visualized under an ultraviolet light and images were taken with a Kodak camera Gel Logic 1500 (Eastman Kodak, USA).

## Statistical Analysis

The statistical package for the social sciences (SPSS), version 9.0 (SPSS Inc., 1988) was used for data analysis. Relationships between traits were investigated using Pearson's correlation coefficients. The Varimax rotation method was used for factor analysis. Cluster analysis was performed based on the morphological traits using Ward's minimum variance method. For genetic analysis, we used the following softwares. The population structure of the 28 genotypes was estimated in the present study. Hardy-Weinberg Equilibrium was used with the *Structure* V2.3.4. software Based on maximum likelihood and delta K (ΔK) values, each band was scored as present (1) or absent (0). The calculation of different dissimilarity functions

was done by package *DARwin 5* version 5.0.158 software (Perrier *et al.*, 2003). The cluster analysis and genetic distance were calculated based on the Dice similarity coefficient. Some parameters were measured to determine the ability of the primers such as polymorphic information content (PIC), resolving power (Rp) and marker index (MI). PIC was calculated by the method of as:  $PIC = 1 - \sum_{i=1}^n (p_i^2)$ , where  $p_i$  is the frequency of bands. PIC for dominant markers was a maximum of 0.5 for  $f = 0.5$ . The averaged PIC values of bands are considered as PIC value of each primer. MI of each primer was calculated according to  $MI = PIC \times EMR$ ,  $EMR = \frac{\text{Polymorphic bands}}{\text{total fragments}} * \text{Polymorphic bands}$ .

Software PopGene, version 1.31 (Yeh *et al.*, 1999) was utilizes to estimate dissimilarity matrix and cluster analysis. The observed number of alleles (Na), effective number of alleles (Ne), gene diversity (H) and percentages of polymorphic loci (P), were analysed.

## Results

### Morphological traits

There were high variations for quantitative and qualitative traits between the studied sweet cherry genotypes. According to the results, 39% of genotypes showed a vigorous growth whereas, 25% of genotypes (A2, A6, A15, A22 and A24) showed a weak growth rate. The results also revealed that, A23 genotype showed late flowering habit. Also, 32.14% of genotypes (A5,

A9, A10, A11, A15, A18, A22 and A26) had very firm fruit texture. The dark red skin colour and red flesh colour were observed in 25% and 28.57% of genotypes, respectively. Moreover, 10.71% of genotypes (A26, A15 and A28) showed low pH values, and 46.43% showed low titratable acidity. Fruit size is very important for the sales and A26 genotype produced the largest fruit. Genotypes A26 (38.75 g) and A7 (20.55 g) showed the largest and the most miniature stone fruit, respectively, while 14.28% of genotypes (A7, A12 and A13) showed a high ratio of flash to the core ration.

Factor analysis based on the varimax method rotation indicated that only three first factors together accounted for 80.06% of the total variance (Table 2). First factor, which accounted for about 46.47% of the variation, was strongly

associated with the fruit characteristics. Second factor, which accounted for about 19.18% of the variation was associated with the leaf characteristics. Third factor, which accounted for 14.40% of the variation, was associated with fruit tails traits.

Correlation of coefficient among different studied traits such as fruit weight and fruit flesh weight ( $r = 0.998^{**}$ ), pit weight and pit weight mean ( $r = 0.998^{**}$ ), leaf width and leaf area ( $r = 0.919^{**}$ ), of sweet cherry genotypes revealed significant correlations.

Descriptive analysis of quantitative traits, including mean value, maximum, minimum, range, and coefficient of variations among sweet cherry genotypes showed a relatively high degree of variation (Table 3).

**Table 2.** Eigen value, percent of variance and cumulative variance of extracted factors.

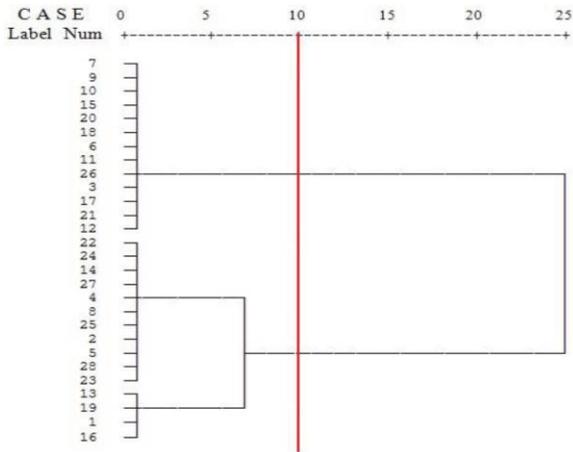
Factors	Eigen value	Variance %	Cumulative variance %
1	7.436	46.475	46.475
2	3.069	19.183	65.658
3	2.305	14.407	80.065

**Table 3.** Minimum, maximum, mean values, standard deviation and coefficient of variations for quantitative traits observed in 28 sweet cherry genotypes.

Traits	Min.	Max.	Mean	SD	CV (%)
Fruit weight	190.20	412.68	266.23	48.54	18.23
Large fruit weight	2.54	5.47	3.828	0.6268	16.37
Mean fruit weight	1.90	4.12	2.658	0.4851	18.7
Pure flash weight	168.35	373.93	240.41	46.80	19.46
Fruit length	12.97	18.78	15.19	1.145	7.54
Fruit width	12.54	16.75	14.14	1.051	7.43
Pit weight	20.55	78.75	25.84	3.360	14.04
Mean pit weight	0.20	0.39	0.2579	0.0366	14.19
Fruit tail weight	6.32	13.68	8.639	1.560	18.05
Fruit tail length	3.70	5.83	4.590	0.5009	10.89
Leaf area	2289	4234.11	3015.23	63.82	18.70
Leaf length	8.43	11.93	10.01	0.9211	9.19
Leaf Width	4.62	6.52	5.510	0.5749	10.42

Maximum and minimum coefficients of variations for quantitative traits among the genotypes were recorded in pure flesh weight (19.46 g) and fruit width (7.43 g), respectively. Cluster analysis of the 28 selected genotypes basis on morphological traits was used to estimate the relationships between the selected genotypes in a dendrogram (Fig. 1). Based on this technique, genotypes were classified by the genetic distance 10 into two different groups.

The first cluster consisted of A3, A6, A7, A10, A9, A11, A15, A12, A17, A18, A20, A21 and A26 genotypes that include 36% of genotypes. This group was superior to other groups in terms of fruit and leaf traits. The second cluster divided into two sub-cluster; The first sub-cluster included of A2, A4, A5, A8, A14, A22, A23, A24, A25 and A27 genotypes; and the second sub-cluster consisted of A1, A13, A16 and A19 genotypes.

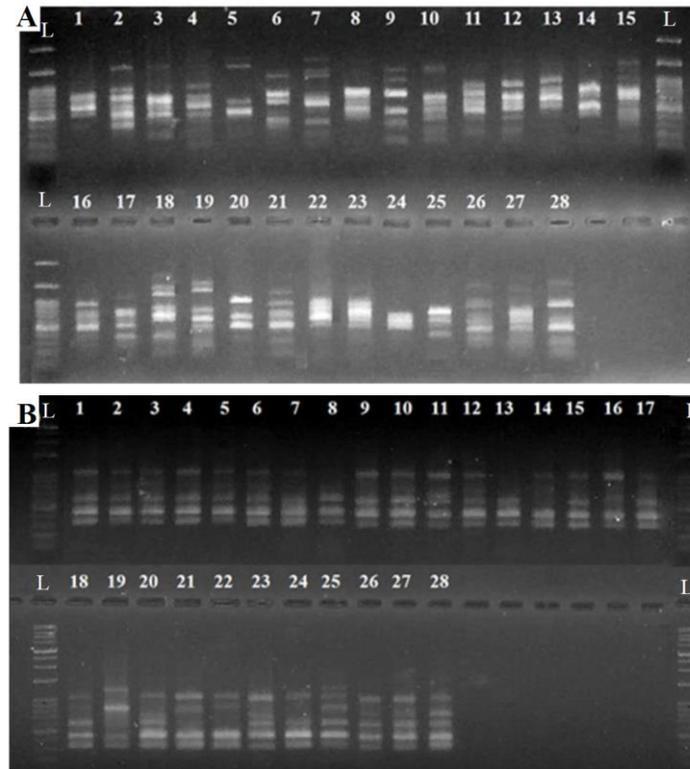


**Fig. 1.** Classification of sweet cherry genotypes based on morphological traits.

**SCoT analysis**

The twelve SCoT primer sets applied to examine twenty-eight genotypes of sweet cherry (Fig. 2). The sixty-nine bands were detected among 28 sweet cherry genotypes, of which 64 were polymorphic (Table 4).

The number of polymorphic bands ranged from 2 (SCoT2) to 9 (SCoT7) with an average of 5.3 per primer. Polymorphism rate ranged from 75 to as high as 100 with an average polymorphism of 89.9%. PIC values ranged from 0.53 (SCoT5) to 0.86 (SCoT7), with an average value of 0.72 per primer. Values for primers' MI ranged from 0.73 to 7.74 the highest MI observed in SCoT7 primer. The primer resolving power (Rp) varied from 3.28 to 8.07. The highest Rp value belonged to the primer SCoT8, whereas primer SCoT2 had the lowest one. The number of observed alleles varied from 5 (SCoT2) to 18(SCoT7), with a mean value of 11. The number of effective alleles from 3.82 for SCoT 2 to 12.38 for SCoT7, with an average value of 8.27 for all primer studied. The Nei's gene diversity for primer ranged from 0.17 for SCoT5 to 0.36 for SCoT 8, with an average of 0.26 (Table 4).

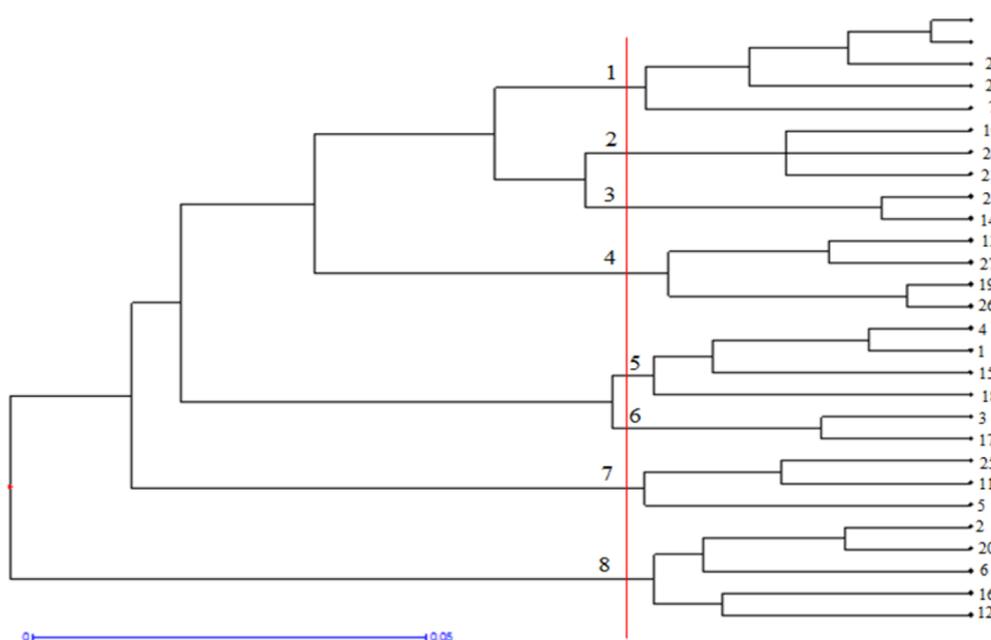


**Fig. 2.** Amplification profile obtained with: A) SCoT7; B) SCoT12 for 28 sweet cherry genotypes; Lane L is 100 bp ladder.

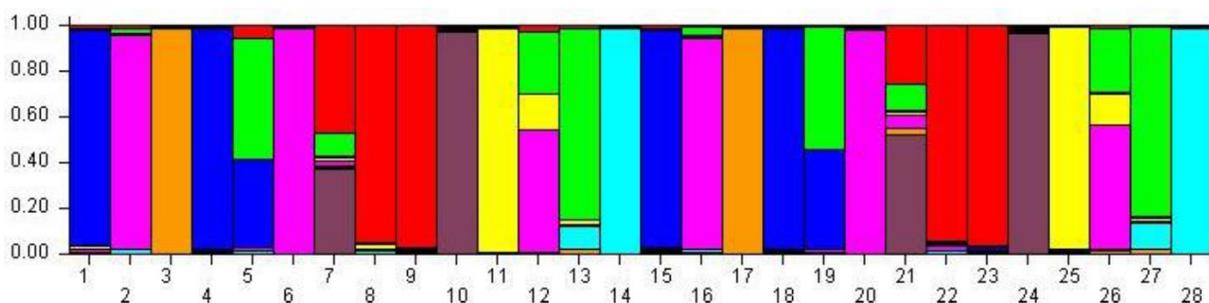
Dice coefficient was used to calculate the genetic similarity of each pair of genotypes. The minimum coefficient (0.65) was observed between A27 and A28 genotypes while; the maximum coefficient (0.14) was observed between A19 and A15. The data obtained from SCoT analysis were used to perform genetic similarity analysis among the 28 sweet cherry genotypes. Based on cluster analysis and Dice similarity coefficient, the 28 sweet cherry genotypes divided into eight groups (Fig. 3). The first cluster consisted of A8, A7, A22, A23 and A9 genotypes, the second cluster included A10, A24 and A21 genotypes, the third cluster consisted of A28 and A14 genotypes, the fourth cluster involved A13, A27, A19 and A26 genotypes, the fifth cluster consisted of A4, A1, A15 and A18 genotypes, the sixth cluster

consisted of A3 and A17 genotypes, the genotype numbers of A25, A11 and A5 to bring into the seventh cluster, as well as the eighth cluster consisted of A2, A20, A6, A16 and A12 genotypes.

The structure analysis revealed the presence of eight populations (Fig.4). Each genotype is represented by a vertical bar partitioned. When structure was run on sweet cherries genotypes, the more probable number of populations was 8, with groups in correspondence with cluster analysis. According to the structure results, blue= A1, A4, A15 and A18, yellow= A11 and A25, red= A7, A8, A9, A22 and A23, pink= A2, A6, A12, A16, A20 and A26, green= A5, A13, A19 and A27, orange= A3 and A17, amaranth deep purple= A10, A21 and A24, aqua = A14 and A28.



**Fig.3.** The dendrogram of 28 sweet cherry genotypes by UPGMA method.



**Fig. 4.** The results of the BAPS analysis of sweet cherry. Each vertical bar represents one individual genotype. Each color represents a genetic group.

## Discussion

In the present study, twenty morphological traits and twelve SCoT markers were employed for genetic diversity evaluation. Based on morphological traits results, due to the weak growth habit of A2, A6, A15, A22 and A24 genotypes, it is more suitable to use them as low growth genotypes and dwarfing rootstock in the breeding programs. A23 genotype was late flowering; therefore, this genotype can be used as late flower genotypes for overcoming freezing injury in the early spring. Skin colour is one of the important parameters that are used to test quality and optimum stage of maturity in fresh sweet cherries (Perez-sanchez *et al.*, 2010). Skin colour changes in sweet cherry fruits is a maturity index used to decide the harvest time (Drake and Elfving, 2017). A5, A9, A10, A11, A15, A22 and A28 genotypes showed the dark red skin color. The fruit weight in sweet cherries is strongly depends on the genetic and the crop load (Goncalves *et al.*, 2006). In this study, A26 genotype produced the largest fruit.

According to the UPGMA dendrogram, the sweet cherry genotypes clustered into two main groups. The first study demonstrated that quantitative and qualitative characteristics are effective for evaluation of genetic diversity between sweet cherry genotypes at the Afil region in the east of Ahar County. The genotypes placed separately in the same group, have the greatest similarity and showed most remarkable difference from the other group (Fig. 1). The high variability in morphological traits has been reported for sweet cherry trees from different studies (Moghaddam *et al.*, 2013). Rakonjac *et al.* (2014) found cluster analysis of morphological variability in wild cherry accessions from Serbia distinguished into two distinct groups. In this grouping, the fruit traits such as fruit length and width, fruit weight, and skin flesh were predominant. El Baji *et al.* (2021) reported that the cluster analysis obtained from morphological traits clearly separated sweet cherries from sour cherries genotypes. In separation of this genotypes, fruit traits such as, weight and width, firmest fruits and short stalk were predominant. Similar to above findings, in this study, results showed that quantitative traits of fruit had vital role in classification of

genotypes. The morphological traits frequently depend on the environment, while, molecular analysis is not affected by environmental factors and can be used for detection of differences between genotypes on the genomic level (Di Vaio *et al.*, 2015). Based on molecular analysis results, this study showed that SCoT markers are a good and reliable molecular tool for analysing genetic diversity and relationships in sweet cherry genotypes. Based on the results, 89.9% of polymorphism was detected by 12 primers. The level of polymorphism (89.9%) was higher in AFLP marker employed by Yan in the sweet cherry (21%) (Struss *et al.*, 2003) and also, was higher in ISSR marker employed by Jamali *et al.* (2020) in the dog rose (*Rosa canina* L.) (%77). The Rp and MI primers are used for identification of primers with high discrimination ability (Zhi-Hui *et al.*, 2014) that in this study, Rp and MI ranged between 3.28-8.07 and 0.73-7.74, respectively. The average Rp and MI value obtained for the SCoT primers used were 5.07 and 3.78, respectively. The SCoT1, SCoT4, SCoT5, SCoT6, SCoT7, SCoT8 and SCoT11 primers with the highest level of polymorphism had more effect to determine genetic distance among other primers. For genetic diversity analysis, the measurement of genetic similarity by molecular markers can provide important information in crop conservation and varietal development (Romero *et al.*, 2009). A19 and A15 genotypes had maximum genetic distance compared with other genotypes. Maybe, the great genetic distance between these genotypes is due to the geographical distance between them that they would help to design breeding programme.

## Conclusion

Our data demonstrate that SCoT marker can distinguish genotypes of sweet cherry even in a population with limited genetic diversity. Results of this study will be useful for conserving and managing sweet cherry genetic resources. Our results showed significant variations in the morphologic and genetic properties of 28 sweet cherry genotypes. The comparison of morphological and molecular clusters showed a moderate correlation between them, for example, genotypes 8, 22, 23 and genotypes 14 and 28 and genotypes 3 and 17 as well as genotypes

20, 12 and 6 and some other genotypes were in the same grouping. The evaluation of genetic diversity based on morphological traits would help the breeder to identify sources of germplasm following by its utilization in breeding programs. In this study, most genotypes did not have suckers, which is a suitable trait in commercial cherry cultivars. Genotype A23 was a late flowering genotype that can be selected as a late flowering parent in breeding programs, especially in the last few years, spring frosts have become more of a rule than an exception. Genotype A13 was also an early season genotype that is an economically valuable. According to morphological and genetic diversity data, we can choose the most suitable genotypes and cultivate them in a separate area as gene bank, select the genotypes with rootstock potential or parents with regard to a particular trait for breeding programme

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#### Conflict of interests

The authors declare that they have no conflicts of interest.

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