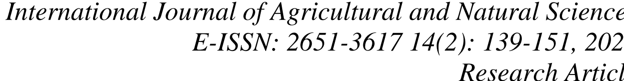

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Research Article

PURPLE CARROT (*Daucus carota* L.) GENETIC DIVERSITY OF CENTRAL ANATOLIA REVEALED BY AFLP AND ISSR

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ABSTRACT. The study was conducted to reveal the genetic diversity of purple carrot in Central Anatolia using AFLP (Amplified Fragment Length Polymorphism) and ISSR (Inter Simple Sequence Repeat) methods. It was aimed to estimate the level of genetic diversity and population structure among purple carrot genotypes. In total, 23 local purple carrot genotypes were collected from Hatay and Konya regions in Turkey. PCR amplification data from 6 AFLP primer combinations generated a total of 148 DNA fragments scored, 138 of which were polymorphic. Also, 12 ISSR primers utilized produced 65 fragments, 50 of which were polymorphic. Molecular data were analysed with the unweighted pair-group method arithmetic average (UPGMA) and principle coordinate analysis (PCoA). The UPGMA analysis demonstrated that the genotypes had a similarity range from 0.54 to 0.94. According to dendogram, purple carrot genotypes generally have two main branches. Structure analysis of the population using DNA markers resulted in 4 distinct subpopulations (K = 4), two of which were represented by single genotypes while the majority of the genotypes were accumulated within a specific subgroup.

Keywords: *Daucus carrota,* purple carrot, genetic diversity, AFLP, ISSR

INTRODUCTION

Carrot (*Daucus carota* L.) is an important member of the *Apiaceae* family cultivated worldwide and used as the source of vitamin A [1]. It is also rich in various vitamins, minerals, carotenoids and phenolic substances [2, 3]. Many characteristics affect carrot quality, nutritional value, color, taste and aroma. In carrot roots, sugar and carotenoid contents are important quality parameters. Β-Carotene accounted for large amount of the total carotenes (44-79%) [4, 5].

Due to its high antioxidant activity and nutrient content, increased interest in colored carrots has recently been reported [6, 7, 8]. Especially purple carrots have higher antioxidant capacity and antioxidant content than the other colors [9, 10]. Purple carrot is grown in Middle Asia, in Europe and Far East [11, 12].

Carrot has an annual production of 571300 tons in 10000 hectares in Turkey [13]. Carrot is cultivated intensively in Central Anatolia of Turkey. Approximately 356000 tons of carrots are grown in the 5000 hectares area of Konya province, an important place in Turkey for carrot [14.] Recently, new varieties have begun to be developed, however the purple carrots in Turkey are in the form of local populations well-adapted to the region and great variation in shape, size, color intensity and efficiency exist.

However, the product is valuable in international markets (i.e., as food coloring and rich antioxidant content), due to the cultivation with local populations, major production problems exist, including early flowering. By focusing on these problems, since the quality and quantity of the produce from the unit area will be increased, purple carrot farming in this unique region will probably become profitable, leading to a considerable expansion of total production areas. Thus, this study is expected to serve as a valuable tool to identify the genetic diversity of the purple carrot genotypes in Turkey for guiding the breeding studies that will lead to superior seeds.

Genetic variation in populations can be defined using morphological and molecular markers. Characterization studies with morphological markers in carrots are also available [15]. However, morphological characters may be influenced by environmental conditions. Molecular marker methods based on DNA sequence polymorphisms are advantageous in determining genetic diversity by interpreting the level of polymorphism regardless of ecological factors [16]. Among the molecular markers, AFLP (amplified fragment length polymorphism) that uses selective amplification of restriction fragments is an important DNA fingerprinting method [17]. Its preference generally depends on its applicability without prior DNA sequence information and due to a high multiplex ratio [18]. In addition, it has a higher sensitivity compared to RAPD and produces large number of loci per reaction compared to other techniques. However, it is more intensive and expensive than the other methods, while it has the potential to be quite useful in genetic analysis [19]. Among PCR based molecular markers ISSR (Inter Simple Sequence Repeats) is also a preferred method without prior DNA knowledge requirement with advantage of simplicity (typing on agarose gel) and cost effectiveness [20]. ISSR has been efficiently used for genetic relationships analyses in diverse species [21, 22, 20].

The purpose of this study was to determine the variation and genetic diversity among the Central Anatolian purple carrot genotypes in Turkey using molecular marker data.

MATERIALS AND METHODS

Plant material

In this study, purple carrot genotypes collected from different regions of Turkey (Hatay, Konya-Center, Konya-Cumra, and Konya-Eregli) were used as material. Planting of 23 different genotypes in a greenhouse of Selcuk University, Faculty of Agriculture was done as 10 individuals from each genotype. Leaf samples were taken for DNA isolation at the 2-3 true leaf stage under appropriate maintenance conditions.

DNA extraction

Genomic DNA was isolated from young leaves of each purple carrot genotype using a modified CTAB method [23, 24]. The quality of DNAs was detected using 1% agarose gel electrophoresis. In addition, DNA samples were also quantified using NanoDrop 1000 at wavelengths of 260, 280, 230 nm.

AFLP Analysis

The AFLP marker technique was performed as described originally by Vos et al. (1995) [17] with minor modifications using fluorescently labelled primers and Li COR / Biosciences, NEN® Model 4300. Genomic DNAs were double digested with 1.5 units

each of *Mse*I and *Eco*RI (MBI Fermentas) in a volume of 12.5 µl and incubated at 37°C for 120 minutes. Adaptors of *Mse*I and *Eco*RI were ligated to the resulting fragments using T4 DNA ligase in a volume of 25 µl buffer ligase 1X and incubated for 120 minutes at 20°C. The ligation mixture was diluted 1/10 and 2.5 µl were added to the pre-amplification reaction including AFLP Pre-amplification. Primer mixture included 10X PCR reaction buffer, 5 U *Taq* DNA polymerase in a reagent volume of 25.5 µl. Pre-amplification was realized in Thermocycler (Bio-Rad T100). PCR conditions were, 2 minutes at 95°C followed by 25 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 72°C with a final 4 minutes at 72°C extention step. The pre-amplification mixture was diluted to 1/40 of which 2 µl was added to the selective amplification reaction including 6 µl reaction buffer, 2 µM *Mse*I, dNTPs, Taq DNA polymerase and primers (0.5 µM IRDye 700 labeled *Eco*RI primer, 0.5 µM IRDye 800 labeled *Eco*RI primer) and mixture in reagent volume of 11 µl. After that, selective amplification was realized in Thermocycler with PCR conditions: 12 touchdown cycles (-0.7°C per cycle) of 30 second at 94°C, 30 second at 65°C, l minute at 72°C; 24 cycles of 30 second at 94°C, 30 second at 56°C, 1 minute at 72°C and 10 minutes at 72°C.

As indicated in Table 1, different primer combinations were used to produce AFLP fragment and contained twelve *Eco*RI primers and six *Mse*I primers. The PCR products were separated by vertical electrophoresis in polyacrylamide gel (6.5%) using the DNA Analyzer.

ISSR analysis

In this study, 24 ISSR primers were pretested where the best 12 primers producing scorable and repeatable bands were determined and the PCR amplifications were continued with these selected 12 primers(Table 1). PCR reaction mix was prepared in a 15 µl volume; including 1.5 µl *Taq* buffer (10mM Tris–HCl, 15mM MgCl2), 1.2 µl of 2.5mM dNTPs, 0.2 µl of 3 unit of *Taq* DNA polymerase, 2 µl (20 ng) of template DNA and 1 µl (5 pM) each of primers. PCR reaction conditions followed were 3 minutes denaturation at 94°C, followed by amplification reactions with 35 cycles at 94°C for 1 minute, at annealing temperature for 50 seconds and 72°C for 1 minute in thermocycler. The PCR products resolved on 1.5% of agarose gel using 1x TBE buffer were imaged under UV light in the presence of ethidium bromide.

Data analysis

Each AFLP and ISSR band was scored as present (1) or absent (0). The level of similarity between the genotypes was based on the total number of fragments (TNF), number of polymorphic fragments (NPF) and mean polymorphism (MP) produced from each primer combination (Table 2).

For UPGMA (unweighted pair group method arithmetic average), binary data were analysed with NTSYS-pc (Numerical Taxonomy Multivariate Analysis System) version 2.1 software package (Exeter Software, Setauket, NY, USA) [25]. A similarity matrix was obtained based on Dice similarity coefficient.

Table 1. *Primers used in AFLP and ISSR reaction*

Primer	Sequence (5'-3')	Primer TYPE
E1	GACTGCGTACCAATTCAAC	ECORI
E2	GACTGCGTACCAATTCAGG	ECORI
E3	GACTGCGTACCAATTCAAG	ECORI
E4	GACTGCGTACCAATTCAGC	ECORI
E5	GACTGCGTACCAATTCACC	ECORI
E6	GACTGCGTACCAATTCACT	ECORI
E7	GACTGCGTACCAATTCACA	ECORI
E8	GACTGCGTACCAATTCACG	ECORI
E9	GACTGCGTACCAATTCACC	ECORI
E10	GACTGCGTACCAATTCAGG	ECORI
E11	GACTGCGTACCAATTCACA	ECORI
E12	GACTGCGTACCAATTCAGC	ECORI
M1	GATGAGTCCTGAGTAACAA	MSEI
M2	GATGAGTCCTGAGTAACAC	MSEI
M3	GATGAGTCCTGAGTAACTT	MSEI
M4	GATGAGTCCTGAGTAACAT	MSEI
M5	GATGAGTCCTGAGTAACTG	MSEI
M6	GATGAGTCCTGAGTAACTC	MSEI
F2	CTCGTGTGTGTGTGTGTGT	ISSR
M1	AGCAGCAGCAGCAGCAGCG	ISSR
M3	AGCAGCAGCAGCAGCAGCC	ISSR
M7	AGAGAGAGAGAGAGAGAGC	ISSR
M8	ACACACACACACACACACG	ISSR
M9	ACACACACACACACACCG	ISSR
M10	ACACACACACACACACCY-Wobbles	ISSR
F8	GCCGCCGCCGCCGCC	ISSR
M15	CACACACACACACACAAG	ISSR
M16	CACACACACACACACAGC	ISSR
M17	CAGCACACACACACACACA	ISSR
M18	CGTCACACACACACACACA	ISSR

Principal coordinate analysis (PCoA) using MINITAB-19 software was performed to observe relationships among the purple carrot genotypes and appoint the number of clusters. Population structure was analysed using STRUCTURE software [26]. This method calculates membership coefficients and identifies individuals by subpopulations based on multiple DNA markers [27]. Cluster analysis was used to determine K value (K = 1-10) where K indicates the number of subpopulations. Several statistical methods have been identified to allow the user to determine which value of K data is best [28]. Analysis was performed using 50,000 iterations and 50,000 burn-in period options for each population (K). For each K number from 1 to 10, five calculations were realized and from these calculations the likelihood values were obtained which were averaged for each K number.

RESULTS AND DISCUSSION

The genetic variation among the 23 Central Anatolian (Turkey) carrot genotypes in the study was investigated using the AFLP primer combinations and ISSR primers by determining phylogenetic relationships. A dendrogram was composed from the data using the UPGMA method from NTSYSpc software. The total number of fragments (148), number of polymorphic bands (138) and the polymorphism ratio obtained from

the data of AFLP analysis are given in Table 2. The overall polymorphism rate of alleles was calculated as 93%. The least number of bands (15) were produced by the E-AAC / E-AGG M-CAA primer combination, while the E-ACA / E-AGC MCTC combination produced the most polymorphic bands (48) in terms of the total number of alleles obtained.

The total number of bands (65), number of polymorphic bands (50) and the polymorphism ratio (%74) obtained from the ISSR analysis are given in Table 2. M10 and M16 primers were found to be the most polymorphic ISSR markers producing 100% polymorphism. The similarity between the purple carrot genotypes according to the dendrogram produced by the Dice similarity coefficient ranged from 0.54 to 0.94 (Fig. 1). According to dendrogram, purple carrot genotypes generally have two main branches. The first one is represented by the genotype 20 which is located at a remote location from all other samples. The similarity coefficient for this sample was 0.54. The second branch is also divided into two main branches. While the 11th genotype was found in one of these branches, the other group had found the accessions of different purple carrot genotypes. The 11th genotype's similarity coefficient was 0.58 and was located at a much closer genetic distance than the 20th genotype (Fig 1). Genotypes have brought subgroups according to their genetic similarities. It was observed that genotypes 2 and 3 could not be separated from each other. However, similarity coefficients of the genotypes 2, 3, 10, 18, 19, 5, 22, 9 were found to be over 0.90 and it can be interpreted that these genotypes constitute a group. In addition, genotypes 1 and 21 showed less similarity to the population, respectively, and the 20th genotype was found to be the furthest.

Table 2. *Polymorphism rates of different primer combinations*

Primer Combination	Total number of fragments (TNF)	Number of polymorphic fragments (NPF)	Mean polymorphism (MP)
1.E-1 /E-2 / M-1	15	13	%87
2.E3/ E-4/ M-2	23	23	%100
3.E-5/ E-6/ M-3	23	18	%78
4. E7 / E-8/ M-4	23	20	%87
5.E-9/ E-10/ M-5	16	16	%100
6.E-11/ E-12/ M-6	48	48	%100
7. F2	8	7	%87
8. M1	6	4	%67
9. M3	5	4	%80
10. M7	5	3	%60
11. M8	4	3	%75
12. M9	6	4	%67
13. M10	2	2	%100
14. F8	5	4	%80
15. M15	8	7	%87
16. M16	9	9	%100
17. M17	4	2	%50
18. M18	3	1	%33
Total	213	188	%80

The dendrogram results obtained from AFLP and ISSR analysis showed that the Central Anatolian originated purple carrot genotypes had high level of genetic variation. This variation is thought to be possibly related to the flower structure of the carrot and

its foreign fertilization. Some other studies have also been conducted to determine genetic diversity in carrots. In the study conducted by Ipek et al. (2016) [29], with simple sequence repeat (SSR) markers, they found a large variation in samples collected from Konya-Eregli region and reported that the genetic similarity varies between 0.20 and 0.70. Also, in another study in which genetic diversity was detected, polymorphism of SSR loci was reviewed in a population of 88 carrot (*Daucus carota* L.) genotypes from North America, Asia and Europe. In this study, the two clusters of 17 and 61 accessions separated from each other formed the individual subgroups of the Asian and the Western type accessions, respectively. Genetic diversity of Asian carrot gene pool is stated to be higher than that of Western carrot gene pool [10]. Similarly, it was reported that accessions originating from Europe and Asia had higher variation and more allelic variants than those from Japan and USA [30]. Similarly, in another study, Grzebelus et al. (2014) [31] employed a Diversity Arrays Technology (DArT) for 65 wild and 94 cultivated carrots and used it to determine genetic variation and to advance a saturated genetic linkage map of carrot. Thus, 900 DArT marker set was analyzed in a collection of plant material including carrot accessions. Also, the accessions were divided into three groups; wild, Eastern cultivated and Western cultivated. In addition, Iorizzo et al. (2013) [32] designed and screened 4000 single nucleotide polymorphisms (SNPs) in a wild and cultivated carrots population to analyze their domestication origins and geographical substructure. Eighty-four genotypes were used, including cultivated and wild carrots. Structure and phylogenetic analyses have shown a clear distinction between cultivated and wild carrots as well as between eastern and western cultivated carrots. In addition, wild carrots from North America were found to be similar to European wild plants.

Bradeen et al. (2002) [33] that used AFLP, ISSR and mitochondrial markers in a study, determined that AFLP and ISSR showed remarkably reliable and reproducible results. According to their results, although both the cultural and the wild-type carrot forms were crossed freely, both groups were distinctly separated from each other by showing a different clustering. Using genetic material containing five commercial and wild forms from *D. carota* L., it was emphasized that there is a rich genetic diversity in carrot and they are relatively irregular in nature. In a study by Shim and Jørgensen (2002) [34], genetic variation within and among five cultivated and five Danish populations of wild carrot varieties was investigated using AFLP markers. They reported that genetic diversity was the largest in the wild carrots and wild carrots were probably exceeded because of the known breeding habit of protandry [35]. Similarly, [30] stated allelic richness and variability in landraces was higher than in open-pollinated cultivars and F1 hybrids. They conducted a study that Intra-population diversity of 18 cultivated carrot of different origins was evaluated based on SSR markers and was reported that the genetic diversity was due to the allelic variation among plants, total variation being 62% within populations and 38% between the populations.

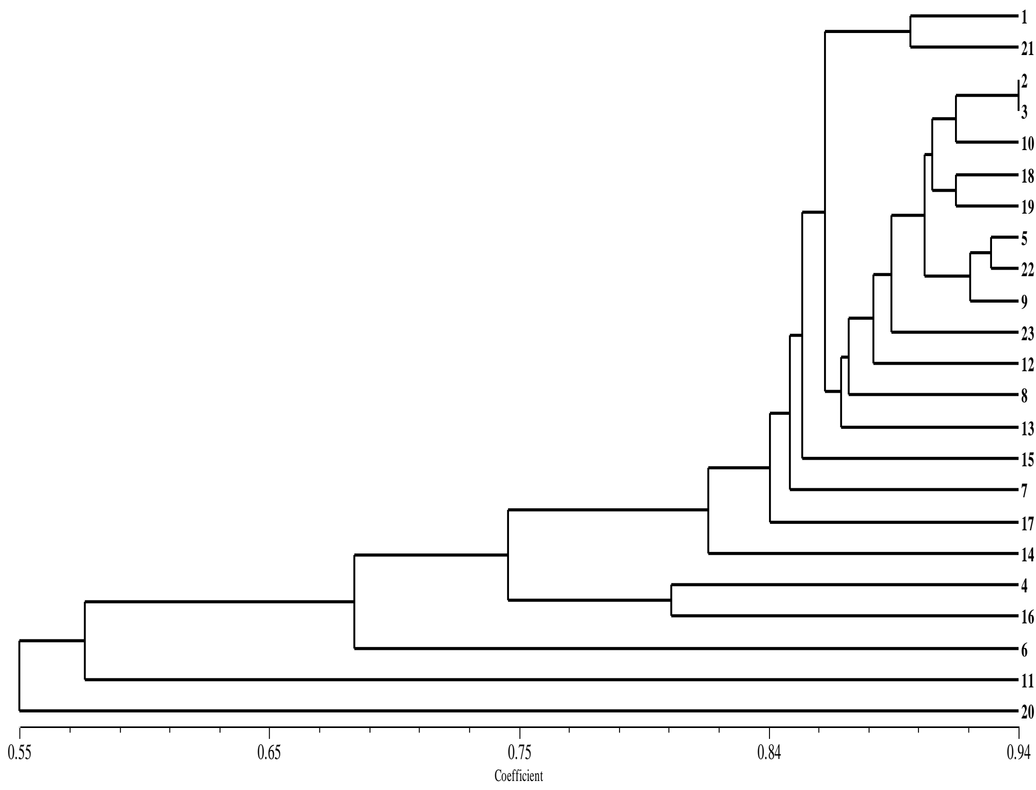

Fig. 1. *The UPGMA dendrogram based on Dice similarity matrix*

In the present study, the purple carrot genotypes clustered in four distinct groups according to the STRUCTURE analysis. Using DNA markers, the number of subpopulations (K) was determined to be four (K = 4). Membership coefficients of 23 genotypes to subpopulations were also determined (Table 3). The first, second, third and fourth subpopulations were composed of 1, 4, 17 and 1 genotypes, respectively. According to the bar plot graph produced from structure analysis, different colors represent gene pools (Fig. 4). Genotypes 4, 11, 16 and 20 were included in the same (second) subpopulation and genotype 6 was in the first group alone (Table 3). Also, Principle Coordinate Analysis (PCoA) was performed to reveal genetic differences between lines with MINITAB-19 software. It showed results supporting the structure analysis. According to the graph, genotype 20 is located far from the others, while 11, 6, 4 and 16 are included in a separate cluster. Other genotypes were clustered at a more remote location (Fig 2). In previous studies, the number of subpopulations in carrots was generally determined to be 3 [10, 29]. In this study, the use of two different molecular marker methods and the different regions defined in these loci (regions restricted by repeat motives in ISSR and specific restriction digestion sites, namely *Mse*I and *Eco*RI sites, in AFLP) may have played a role in obtained 4 subpopulations. In line with the previous studies, the number of individuals included in the 1st and the 4th populations is very small, represented by single samples, and the remaining genotypes were heavily included in the 3rd subpopulation, yet the variation observed in this study may provide agronomically important useful traits.

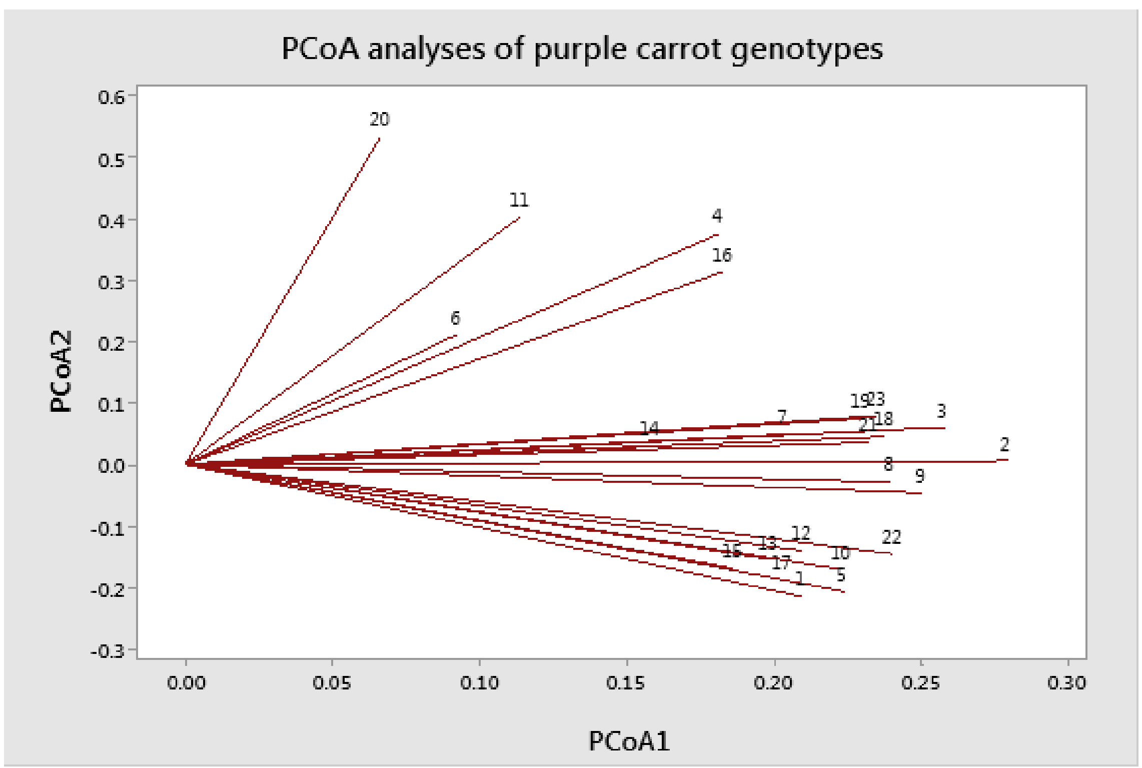

Fig. 2. *The PCoA analyses of purple carrot genotypes*

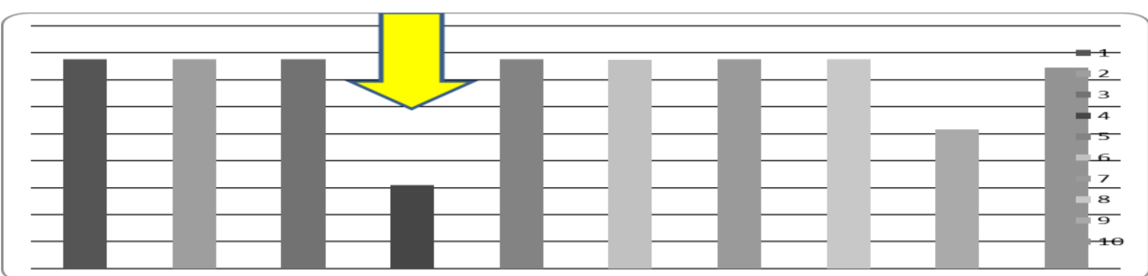

Fig. 3. *Number of Sub-populations*

Table 3. *Sub-population membership coefficients of individuals*

	1	**2**	**3**	**4**
1	0.002	0.001	0.620	0.377
2	0.002	0.000	0.997	0.001
3	0.002	0.004	0.991	0.003
4	0.035	0.602	0.043	0.319
5	0.010	0.001	0.987	0.003
6	0.997	0.001	0.001	0.001
7	0.164	0.075	0.553	0.209
8	0.100	0.115	0.778	0.007
9	0.019	0.002	0.976	0.002
10	0.002	0.002	0.990	0.007
11	0.004	0.982	0.004	0.011
12	0.002	0.007	0.989	0.002
13	0.006	0.002	0.768	0.224
14	0.002	0.002	0.296	0.701
15	0.002	0.005	0.695	0.298
16	0.006	0.798	0.114	0.082
17	0.006	0.002	0.609	0.383
18	0.002	0.001	0.997	0.001
19	0.001	0.001	0.996	0.002
20	0.001	0.995	0.001	0.003
21	0.005	0.006	0.720	0.269
22	002	0.002	0.994	0.003
23	0.007	0.008	0.884	0.101

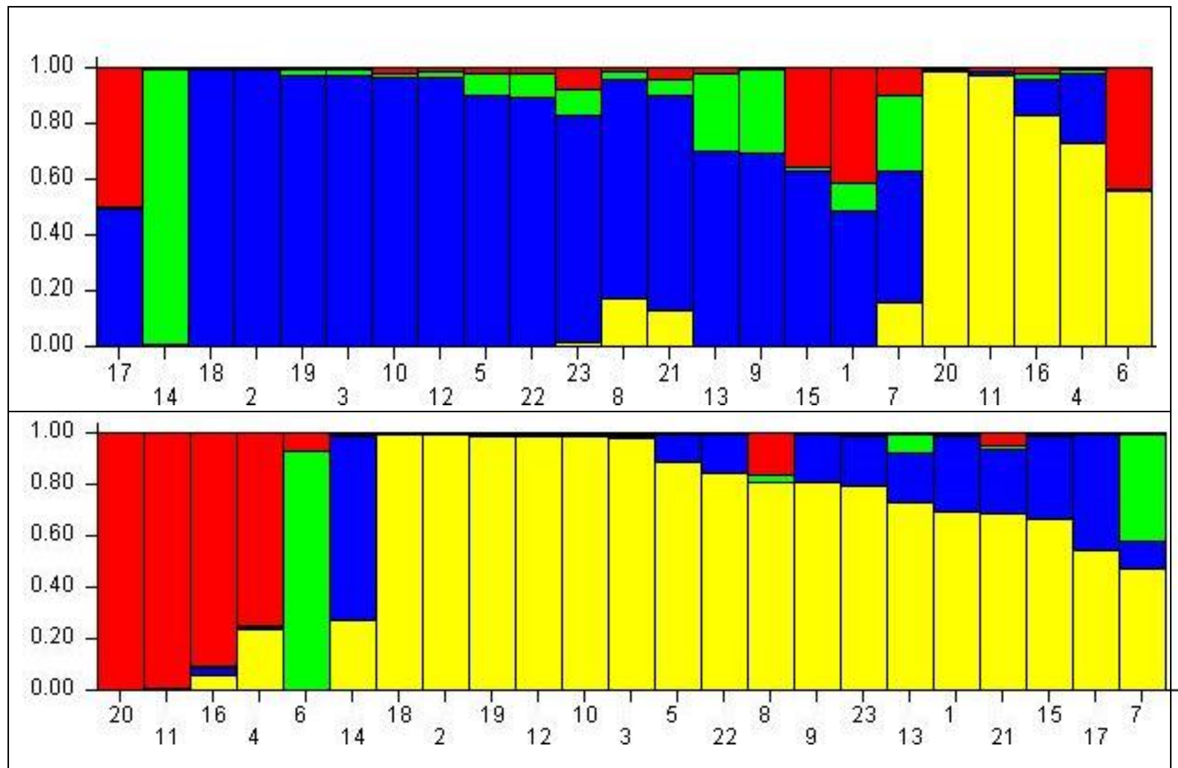

Fig. 4. *Graphical representation of membership coefficients obtained from STRUCTURE software*

All these results support a high level of genetic diversity in the region (Central Anatolia) where this study is conducted. Considering there is a neglect on the utilization of the local diversity, it is important to start breeding activities on the local purple carrot populations that provide variations in shape, size, color intensity and agronomic efficiency.

In conclusion, the presence of well adapted local purple carrot genotypes representing a high level of genetic variation, may serve for the carrot breeders as a rich gene source to enhance the production capacity of the farming population of Central Anatolia while it will also support globally the supply chain of valuable food coloring reagents as well as the improved quality trade reagents like antioxidants. It is thought that there is no genetic bottleneck due to the genetic diversity of carrot genotypes in Central Anatolia. Hence, the collection and characterization of the populations in this common area of purple carrot production will contribute substantially to future breeding efforts. In this context, it is seen as a necessity to initiate country-based purple carrot breeding programs before becoming suppressed by the widespread distribution of extrinsic F1 hybrid purple carrot varieties currently absent in the region. As a matter of fact, genetic erosion in herbaceous plants can reach extreme dimensions with the spread and domination of high-quality varieties in a region. Foreign-pollination nature of this biennial plant is limiting the effectiveness of the selection and prolongs the breeding period.

In addition, due to the color pigments it contains purple carrot, today, has an intense usage industrially but also it is an important vegetable that is widely used in the field of health with its high antioxidant properties. Therefore, this study has genetically revealed

the diversity of the gene pool in the Central Anatolia and constituted a source for future studies.

Based on this study data, future studies related to the morphological and biochemical properties of purple carrots can be planned, and purple carrot breeding programs with high antioxidant properties and suitable for the industry can be created. If the purple carrot breeding can benefit from biotechnological methods, this can be an advantage for the breeding process.

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