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Full Length Research Paper

Molecular diversity among Turkish oaks (*QUERCUS*) using random amplified polymorphic DNA (RAPD) analysis

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The genus *Quercus* (Fagaceae) includes the most important woody plants with decidious and evergreen species in Northern hemisiphere. They have a problematic taxonomy because of widespread hybridization between the infrageneric taxa. Turkey is one of the most important region of the world according to oak species number and variation. In this study, species belonging to evergreen oaks in Turkey were investigated to solve taxonomic problems and to design the limit of taxa by using random amplified polymorphic DNA (RAPD) data. Here, three species of evergreen oaks known as *Quercus coccifera*, *Quercus ilex* and *Quercus aucheri* were studied in all area located and made the comparison within and among species studied using ten RAPD markers. As a result; it can be stated that the presence of the three species in *Ilex* section is clear. Furthermore, existence of two infraspecific taxa or two seperate taxa in species level within *Q. coccifera* may be quite possibly considered.

Key words: *Quercus ilex*, *Quercus coccifera*, *Quercus aucheri*, random amplified polymorphic DNA (RAPD).

INTRODUCTION

The genus *Quercus* is one of the most diversified groups of the trees of temperate zone in north Hemisiphere with more than 500 species (Govaerts and Frodin, 1998; Tovar-Sanchez and Oyama, 2004; Olfat and Pourtahmasi, 2010; Maryam Ardi et al., 2012). Govaerts and Frodin (1998) state that the genus *Quercus* is represented by 531 species in the world and 250 of these species in America, 125 of these in Asia and remaining species in Europe, North Africa and Macaronesia. The area including South East Asia and Pacific islands is the center of morphological variation of Fagaceae, altough this area does not contain the most species of *Quercus* (Kaul, 1985).

Oaks are the woody, widespread, long-lived, outcrossing and wind-pollination species. For this reason, oaks can spread too wide geographic regions and as a result of this, they show high variations comparison to other woody plant species (Kremer and Petit, 1993; Hokanson et al., 1993; Bacilieri et al., 1996; Neophytou et al., 2010). It is well known that extensive hybridization behaviors may occur among species (Bacilieri et al., 1996; Manos et al., 1999; Samuel, 1999; Jensen et al., 2009; Neophytou et al., 2010) in the same group or section in the genus *Quercus*, because of weak reproductive barriers between oak species. Consequently, hybrid species spring up. Therefore the genus *Quercus* is taxonomically one of the most problematic groups (Bacilieri et al., 1996). The most of species in Turkey and all distributed countries have taxonomic problems. Taxonomic problems can be solved by molecular studies in addition to morphological and cytological studies and so genetic diversity and the limits of taxa can be deter-

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mined more clearly (Borazan and Babaç, 2003; Yılmaz et al., 2008; Simeone et al., 2009; Alam et al., 2009; Papini et al., 2011; Yılmaz et al., 2011).

Turkey is one of the most important region for oaks according to the species number and geographical distribution. Oaks in Turkey have a natural distribution of about 6.5 million ha area represented by 18 species in three different section (Davis, 1982; Yaltırık, 1984; Kasapligil, 1992) as white oaks (*Quercus* L.), red oaks (*Cerris* Loudon.) and evergreen oaks (*Ilex* Loudon.). Here, the species analysed were *Quercus coccifera*, *Quercus aucheri* and *Quercus ilex* known as evergreen oaks. These are very problematic species in Turkey in the comparison to other members of the genus. The distribution area for *Q. aucheri* is only south weast region of Turkey and in the Greek island like Rhodos in the world.

However; *Q. aucheri* is confused with the another member of *Ilex* section, (*Q. coccifera*). As a result, it can be stated that it is not very well known species for bio-systematic features and species limit. Moreover, it is controversial subject that *Q. coccifera* and *Quercus calliprinos* Webb. are seperate species or *Q. coccifera* has two subspecies known as *Q. coccifera* subsp. *coccifera* and *Q. coccifera* subsp. *calliprinos* (Toumi and Lumaret, 2001; Salvatore and Paola, 1976). Distribution area for *Q. calliprinos* is east mediterranean region and seperated from *Q. coccifera* with different living area. These taxonomical problems indicate that the real phenetics and fylogenetics relations within *Ilex* section have not still been fully explained. Hybridization and vegetative variations cause problems and make difficult to determine the borders of taxa.

Random amplıfıed polymorphıc DNA (RAPD) is a polymerase chain reaction (PCR) based technique used to show polymorphism among species. Especially this method is very helpful for systematics purposes and phylogenetic relation. For this aim, RAPD was used in this study as molecular technique (Kumar and Gurusubramanian, 2011). In order to solve this problem, variations within and between populations of taxa were pointed out by using some statistical analyses such as Statistica version 8.0 for principal component analysis (PCA) and cluster analysis (CA) using an unweighted pair group method (UPGMA) analysis and Popgen 32. According to the results of statistical analyses, it was attempted to draw the most possible borders of taxa based on the DNA bands obtained from RAPD analyses (Sesli and Yegenoglu, 2009; Açık et al., 2009; Kavalcıoglu et al., 2010) and a better phenetic classification by using molecular characters showing high correlations with each other.

MATERIALS AND METHODS

Study materials are composed of three species (*Q. coccifera*, *Q. ilex* and *Q. aucheri*) belonging to *Ilex* section of Turkey oaks. Totally 26 populations were represented to show variations within and among species (Table 1 and Figure 1). Leaves were used as material to show the differences in the molecular study. *Q. coccifera* was represented by 16 populations and other two species (*Q. ilex* and *Q. aucheri*) were represented by 5 populations. Especially, fresh and young leaves were preferred as material. Collected leaves were put into plastic bags filled silica gel and dried for the DNA isolation.

DNA extraction

Firstly, leaves in plastic bags filled silica gel were ground in liquid nitrogen using a mortar. DNA was extracted using a DNAeasy Plant Mini Kit (Qiagen). Extracted DNAs were kept at 4°C. Quality of each DNA sample were controlled by running on agarose gel before being used in PCR.

RAPD-PCR and gel electrophoresis

Molecular analysis was performed using RAPD method (Williams et al., 1990; Welsh and McClelland, 1991). Totally 30 primers, studied in oaks previously, were selected to find primers that exhibit polymorphism and give reproducible results. After the initial screening, 10 primers giving the best results among 30 primers were selected for further analysis (Table 2).

Amplification reactions were carried out in a 25 µl mix. The reaction mixture was prepared using PCR Buffer, $MgCl_2$, dNTP mixture containing dATP, dCTP, dGTP and dTTP, 10- base RAPD primer and taq DNA polymerase. After the primer selection, PCR conditions was determined. The program consisted of 40 cycles as fallows: Denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extention at 72°C for 2 min. A final extention at 72°C for 10 min was included.

The amplification products were electrophoresed in 1.4% agarose gels with TBE buffer at 100 V for 1 h and 30 min and stained with ethidium bromide. Gels with amplification fragments were visualized and photographed under ultraviolet light. RAPD bands were estimated by reference to a 100-bp ladder (Fermentas).

Data analysis

In order to score the RAPD products, amplified fragments were recorded as present (1) or absent (0) in all individuals for each fragment. Then the tables were constructed containing number and size of the DNA fragments for each populations. Polymorphic bands were determined for all populations. Molecular diversity among populations and species was evaluated by calculating the percentage of polymorphic fragments. The comparison of genetic distance and genetic similarity were calculated according to Nei (1972). RAPD data were evaluated by using two different statistical programs. Statistica version 8.0 were used for PCA and CA using an unweighted pair group method (UPGMA) analysis. Popgen 32 was used for genetic similarity and genetic distances.

RESULTS

In the RAPD analysis, 156 individuals representing 26 populations were used. A total 217 polymorphic bands were scored using the 10 RAPD primers. The size of the amplication products was between 150 to1600 base-pair. Table 2 shows the total number of polymorphic bands provided from each primers. The minimum and maximum size of amplification products provided from different

Table 1. Populations sampled (C = *Q. coccifera*, A = *Q. aucheri*, I = *Q. ilex*).

Pop. No	Location	Coordinates		Altitude
		N	E	(m)
C1	İzmir-Balıkesir border area, Altınova barrage road	39° 12.903	026° 49.302	70
C2	İzmir-between Dikili-Çandarlı, 20 km. to Çandarlı	39° 01.253	026° 55.505	40
C3	Manisa-between Kırkağaç-Akhisar, 1-2 km. after Çandarlı	39° 05.800	027° 40.257	190
C4	Çanakkale-Ezine-Bozcaada pier	39° 47.950	026° 12.115	50
C5	Gökçeada-between Gökçeada-Dereköy	40° 09.689	025° 49.586	60
C6	Mersin-5-10 km. after Seratvul	36° 50.997	033° 18.402	1400
C7	Karaman-between Mut-Ermenek, 45 km. before Ermenek	36° 37.276	032° 55.182	1300
C8	Antalya-between Korkuteli-Bucak, 25 km. before Bucak	37° 15.582	030° 19.362	920
C9	Aydın-Eski Çine, Ovacık village	37° 32.889	028° 05.310	300
C10	Aydın-Söke, between Bağarası-Akçakaya village	37° 40.350	027° 31.347	40
C11	Muğla-between Muğla-Kale, 59 km. before Kale	37° 08.142	028° 32.157	800
C12	Denizli- between Kale-Tavas, 1-2 km. before Tavas	37° 33.069	029° 03.150	940
C13	Uşak-between Sivaslı-Uşak, 12 km. after Sivaslı	38° 34.259	029° 36.303	825
C14	Gaziantep- between Yavuzeli-Araban	37° 22.975	037° 33.292	740
C15	Kahramanmaraş- between k.maraş- göksun	37° 43.514	036° 40.038	1075
C16	Hatay-between Kırıkhan-Hassa	36° 36.554	036° 23.591	350
A1	Antalya-between Kemer-Kumluca	36° 25.429	030° 25.447	530
A2	Aydın-Çine,Across from the cemetery Kuruköy	37° 33.558	028° 04.047	180
A3	Aydın-Priene-Söke	37° 44.967	029° 16.369	90
A4	İzmir-Selçuk-Zeytinköy	37° 59.569	027° 17.226	65
A5	Muğla-between Milas-Bodrum, Dörttepe village	37° 11.242	027° 37.142	8
I1	Zonguldak-Alaplı, Sabırlı village	41° 08.901	031° 23.147	180
İ2	Zonguldak-between Alaplı-Düzce	41° 08.443	031° 20.596	4
I3	Düzce- between Yığılca-Alaplı	41° 09.136	031° 23.627	60
I4	İstanbul-between Anatolian Fortrees-Kavacık	41° 04.220	029° 05.085	65
I5	Gökçeada-between Gökçeada-Dereköy	40° 09.689	025° 49.586	60

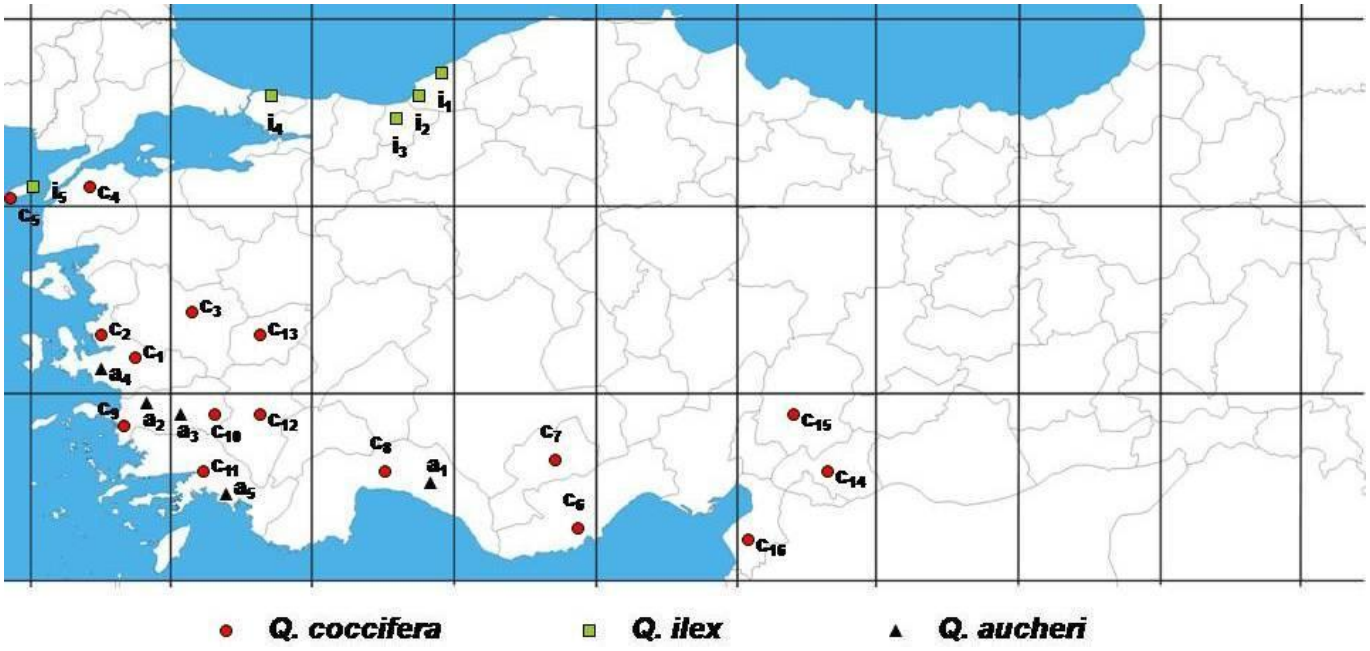

Figure 1. Distribution of studied populations of *Q. coccifera*, *Q. ilex* and *Q. aucheri* in Turkey.

Table 2. The list of primers used in RAPD and analysis of PCR amplification products by selected primers.

Prımer	Sequence (5'-3')	Number of bands	Amplification products (bp)
OPA-01	CAGGCCCTTC	20	300 to 1400
OPA-08	GTGACGTAGG	18	200 to 1400
OPA-09	GGGTAACGCC	21	250 to 1400
OPB-04	GGACTGGAGT	22	200 to 1400
OPX-04	CCGCTACCGA	23	150 to 1400
OPC-03	GGGGGTCTTT	22	150 to 1600
OPC-09	CTCACCGTCC	19	300 to 1300
OPS-09	TCCTGGTCCC	25	200 to 1500
OPS-18	CTGGCGAACT	23	200 to 1400
OPU-01	ACGGACGTCA	24	200 to 1400
Total	10	217	150 to 1600

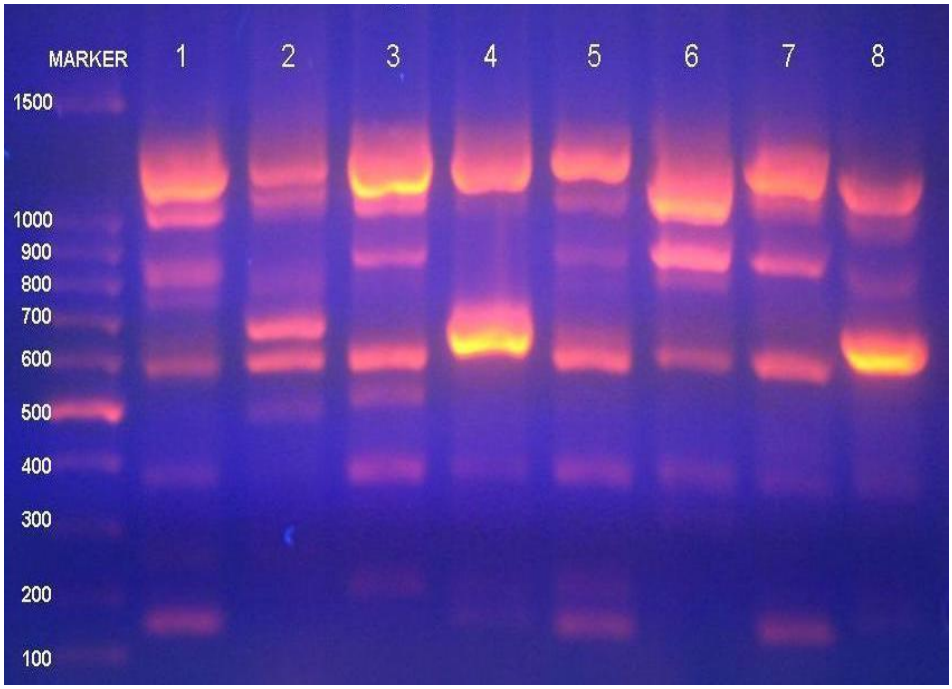

Figure 2. RAPD products in C13 population with OPX-04 primer.

primers were also listed in Table 2.

In order to score the RAPD products, individuals of each population were run separately for each primer (Figure 2). Additionally, RAPD products of six individuals from every population were bulked and run together, to see all populations products in the same gel for each primer (Figure 3). CA and PCA were carried out for the analysis of variations within and among studied species. According to these results, *Q. ilex* and *Q. aucheri* were observed as close two separate groups. Populations of *Q. coccifera* showed more differences than populations of *Q. ilex* and *Q. aucheri*. But fundamentally, three studied species showed differences from each other. When the each species were evaluated separately, generally geographically close populations showed more similarity than geographically distant ones (Figures 4 and 5). Populations belonging to *Q. ilex* were separated into two subgroups in CA pehenogram. The first of these was I1, I2 and I3 populations.

The second sub-group of *Q. ilex* was composed of I4 and I5 populations. Other species, *Q. aucheri* was separated into two sub-groups like *Q. ilex* but here C7 population of *Q. coccifera* showed the high similarity with the populations of *Q. aucheri*. Finally, when the populations of *Q. coccifera* were examined, it drew attention that *Q. coccifera* was separated into three sub-groups. When these three sub-groups are observed attentively, they were separated as geographically from each other

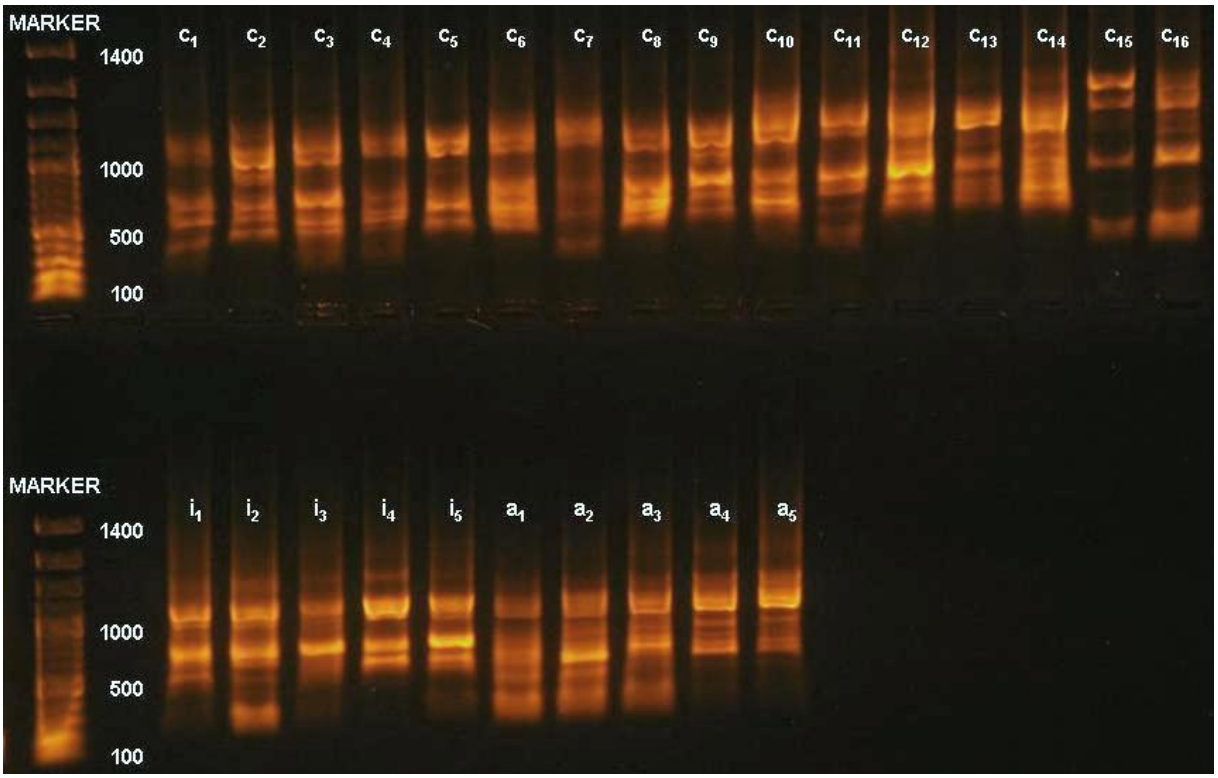

Figure 3. Visualization of all population's RAPD products with OPB-04 primer.

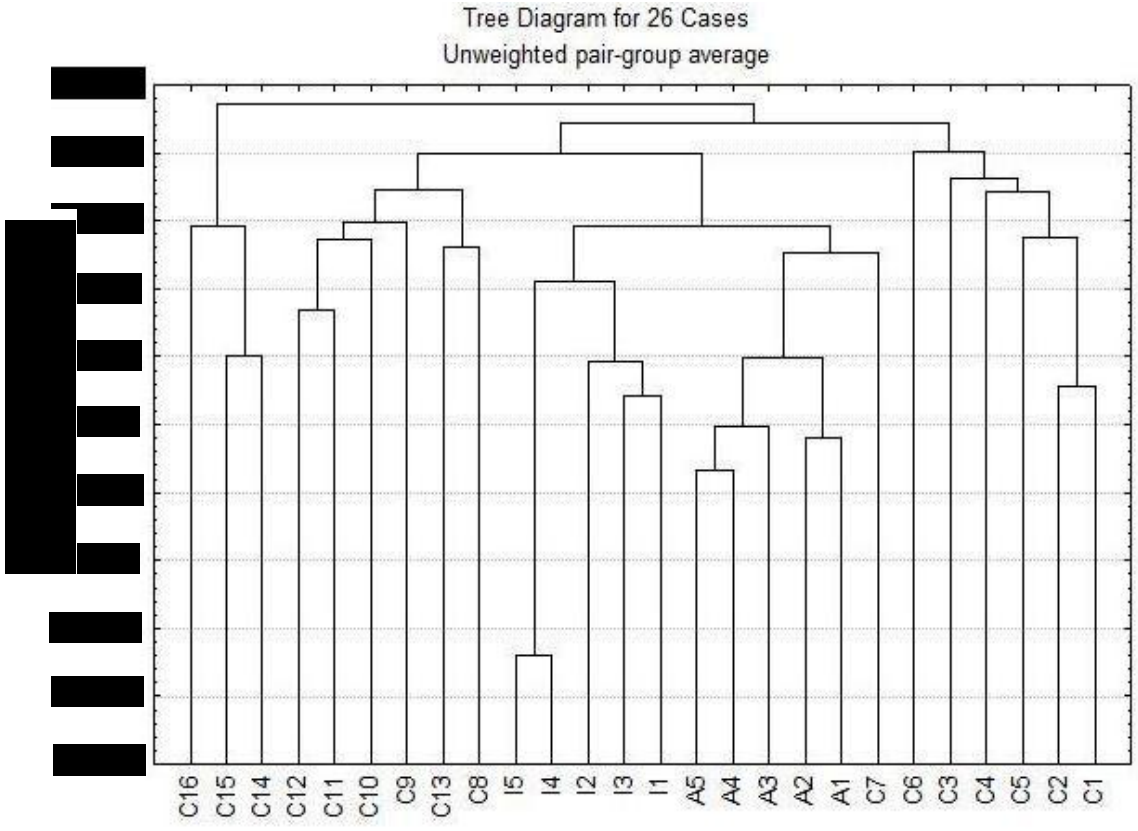

Figure 4. Phenogram resulting from cluster analysis with UPGMA.

Table 3. The comparison of genetic distance (below diagonal) and genetic similarity (upper diagonal) (Nei, 1972).

	c1	c2	c3	c4	c5	c6	c7	c8	c9	c10	c11	c12	c13	c14	c15	c16	i1	i2	i3	i4
c1	***	0.998	0.985	0.991	0.991	0.992	0.993	0.991	0.996	0.991	0.992	0.988	0.990	0.985	0.988	0.986	0.985	0.990	0.987	0.991
c2	0.001	****	0.981	0.990	0.989	0.992	0.990	0.987	0.993	0.987	0.991	0.985	0.987	0.982	0.985	0.983	0.981	0.986	0.982	0.986
c3	0.014	0.018	****	0.971	0.973	0.987	0.986	0.987	0.980	0.987	0.990	0.984	0.982	0.973	0.990	0.989	0.976	0.986	0.987	0.985
c4	0.008	0.010	0.029	****	0.985	0.990	0.989	0.983	0.994	0.985	0.980	0.978	0.982	0.974	0.973	0.977	0.981	0.977	0.984	0.985
c5	0.009	0.010	0.026	0.014	****	0.987	0.990	0.992	0.989	0.987	0.989	0.995	0.993	0.976	0.981	0.981	0.981	0.980	0.985	0.989
c6	0.007	0.007	0.012	0.009	0.013	****	0.990	0.987	0.992	0.987	0.988	0.985	0.987	0.977	0.986	0.986	0.977	0.979	0.985	0.986
c7	0.006	0.009	0.013	0.011	0.009	0.009	****	0.993	0.991	0.989	0.990	0.990	0.990	0.981	0.990	0.990	0.986	0.992	0.989	0.992
c8	0.008	0.012	0.012	0.016	0.007	0.012	0.006	****	0.986	0.991	0.990	0.997	0.994	0.977	0.987	0.989	0.982	0.984	0.992	0.989
c9	0.003	0.006	0.019	0.005	0.011	0.007	0.008	0.013	****	0.992	0.990	0.983	0.989	0.985	0.987	0.983	0.985	0.988	0.987	0.992
c10	0.008	0.012	0.013	0.015	0.012	0.012	0.011	0.008	0.007	****	0.992	0.990	0.996	0.989	0.992	0.991	0.988	0.983	0.985	0.992
c11	0.007	0.008	0.009	0.020	0.010	0.011	0.009	0.009	0.010	0.007	****	0.990	0.990	0.984	0.991	0.988	0.990	0.990	0.988	0.989
c12	0.011	0.014	0.015	0.021	0.004	0.014	0.009	0.002	0.016	0.009	0.009	****	0.995	0.972	0.983	0.986	0.979	0.981	0.988	0.991
c13	0.009	0.013	0.017	0.017	0.006	0.012	0.010	0.005	0.011	0.003	0.009	0.004	****	0.987	0.989	0.993	0.987	0.977	0.983	0.991
c14	0.014	0.017	0.026	0.026	0.024	0.023	0.019	0.022	0.014	0.010	0.015	0.028	0.012	****	0.992	0.989	0.987	0.974	0.965	0.975
c15	0.011	0.014	0.009	0.026	0.019	0.013	0.009	0.012	0.012	0.008	0.008	0.016	0.010	0.007	****	0.993	0.982	0.988	0.979	0.984
c16	0.013	0.017	0.010	0.022	0.018	0.013	0.009	0.010	0.016	0.008	0.011	0.014	0.006	0.010	0.006	****	0.989	0.977	0.979	0.985
i1	0.015	0.018	0.023	0.018	0.018	0.022	0.013	0.017	0.014	0.011	0.009	0.020	0.012	0.012	0.018	0.011	****	0.977	0.981	0.983
i2	0.010	0.013	0.013	0.023	0.020	0.020	0.007	0.015	0.012	0.017	0.009	0.018	0.022	0.025	0.012	0.023	0.022	****	0.986	0.988
i3	0.012	0.018	0.012	0.015	0.014	0.014	0.010	0.007	0.012	0.014	0.011	0.011	0.016	0.034	0.021	0.020	0.018	0.013	****	0.989
i4	0.008	0.013	0.014	0.015	0.010	0.013	0.008	0.010	0.007	0.007	0.010	0.008	0.009	0.024	0.015	0.014	0.017	0.011	0.011	****
i5	0.004	0.007	0.011	0.012	0.008	0.011	0.007	0.005	0.007	0.008	0.006	0.006	0.010	0.026	0.016	0.017	0.017	0.008	0.005	0.005
a1	0.004	0.008	0.020	0.007	0.008	0.013	0.005	0.006	0.006	0.010	0.013	0.010	0.011	0.023	0.017	0.020	0.020	0.010	0.008	0.009
a2	0.005	0.007	0.013	0.014	0.010	0.011	0.009	0.006	0.007	0.010	0.008	0.009	0.013	0.025	0.014	0.021	0.023	0.008	0.007	0.011
a3	0.009	0.013	0.010	0.018	0.014	0.017	0.008	0.005	0.016	0.012	0.009	0.006	0.013	0.032	0.019	0.017	0.018	0.010	0.007	0.008
a4	0.009	0.013	0.027	0.006	0.011	0.015	0.010	0.012	0.007	0.016	0.017	0.014	0.018	0.036	0.027	0.031	0.025	0.014	0.008	0.011
a5	0.003	0.008	0.018	0.009	0.008	0.012	0.006	0.008	0.003	0.010	0.010	0.010	0.011	0.023	0.016	0.020	0.019	0.008	0.006	0.005

(Figure 4).

Populations belonging to *Q. coccifera* evaluated in the West and South West region of Turkey are C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12 and C13 (Figure 1) and results from cluster analysis with UPGMA showed that the populations from these regions were similar in the comparison to the remaining (Figures 4 and 5). Eventually, populations having the highest differences of *Q. coccifera* were C14, C15 and C16. These popu-lations originated from East Mediterranean region (Figure 1). The molecular analysis with CA and PCA revealed a high degree of separation between the species.

When the tables of genetic distance and similarity was investigated, the lowest genetic distance was observed between C1-C2 and A1-A5 populations (Table 3). In other words, the highest genetic similarity was observed between C1-C2 and A1-A5 populations. The highest genetic distance was between C14-A4 and C14-I3 populations, respec-tively. Therefore, the lowest genetic similarity was between C14-A4 and C14-I3 populations.

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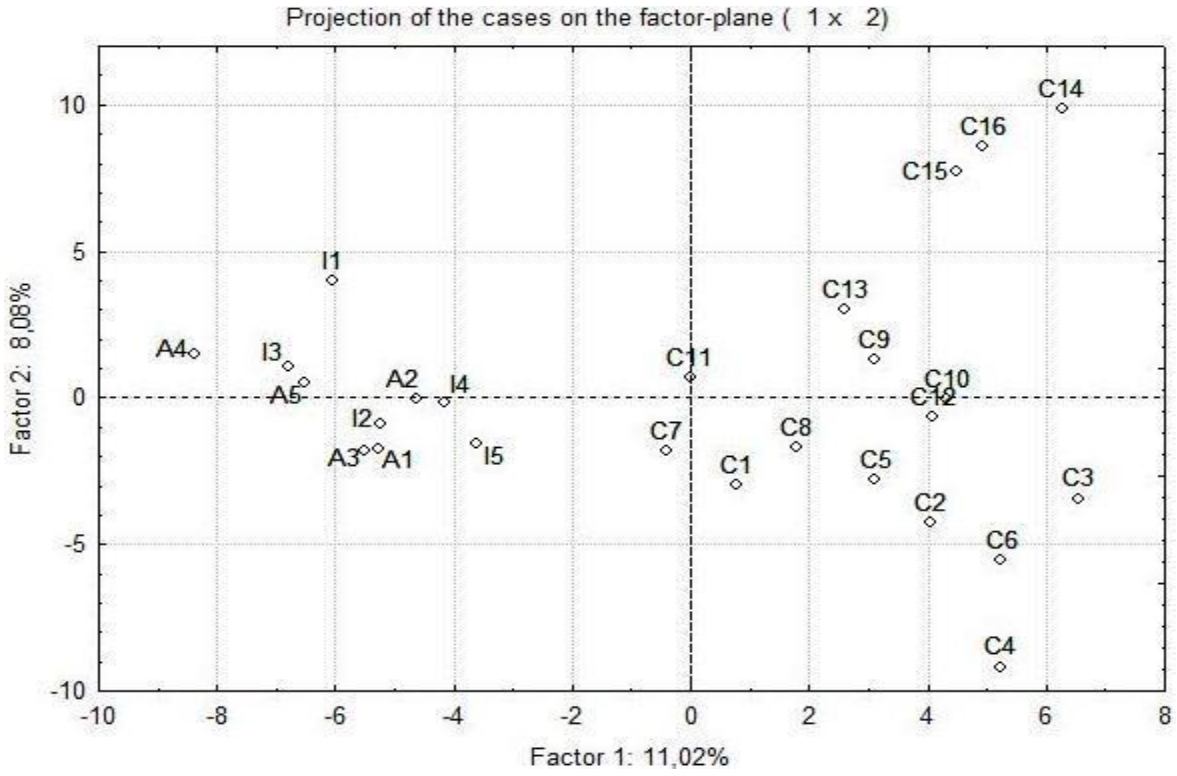

Figure 5. The resulting projection of principal component analysis.

1982).By this study; the lack of molecular properties of *Ilex* section is completed in detail. The results gave the satisfactory findings for phenetic groupings of taxas. The significant differences are found on the all studied species. Firstly, *Q. ilex* is separated from the other species. Due to similarities among the some *Q. coccifera* and *Q. aucheri* populations, they are well separated with each other. This is the first study that shows *Q. coccifera* is separated into two geographical groups in Turkey. The first group has the populations sampled from West and South West regions of Turkey. The populations sampled from C14, C15 and C16 belonging to East Mediterranean region included into the second group. This geographically separation within populations of *Q. coccifera* suggests that there are sub-groupings or different species in this taxon. The most common group is *Q. coccifera* found in many regions, while the less and restricted group is found only in the East Mediterranean region. The second group is geographically closer to Syria, Israel and Palestine. In addition, these two groups are represented as a single *Q. calliprinos* species and its two subspecies as *Q. calliprinos* subsp. *coccifera* and *Q. calliprinos* subsp. *calliprinos* in Flora of Palestine (Zohary, 1966). When the studied populations are compared with each other according to the genetic similarity, it can be said that genetically distant populations are also located geographically in different and far regions (Figures 4 and 5; Table 3). The most high genetic similarity are found between C1-C2 and A1-A5 populations which are also geographically close populations. On the contrary, genetically the most distant populations, C14-A4 and C14-I3 are the two different species which are geographically located very distant.

As a result of this study, it might be suggested that: (1), The results showed the presence of the second group within *Q. coccifera* but this needs to be supported in a study including *Q. calliprinos* samples from Syria, Israel and Palestina; (2), the groupings based on molecular studies support the presence of the three species in *Ilex* section; (3), the two groups showing geographical differentiations within *Q. coccifera* may strengthen the existence of two infraspecific taxa such as *Q. coccifera* subsp. *coccifera* and subsp. *calliprinos* or two different taxa at species level.

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