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Analysis of Genetic Variations within *Q. coccifera* L. and the Effects of Geographical Differences in Relationships among Populations

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Abstract

Aim of study: This study aims to determine the variations within *Q. coccifera* and the effects of geographical differences on the variations among populations.

Area of study: Samples in this study containing *Q. coccifera* populations of Mediterranean countries were obtained from National Center for Biotechnology Information (NCBI) database.

Material and method: In this study, all populations belonging to *Q. coccifera* and *Q. calliprinos* based on rbcL gene and matK gene-partial trnK gene intron sequences, were collected from NCBI database. It is aimed to determine more informative results about the phylogenetic relations of populations and to evaluate the barcoding regions in terms of their abilities to reveal the relationships and variations of populations. For this purpose, Molecular Evolutionary Genetics Analysis (MEGA 11) was performed.

Main results: For both barcoding regions, the presence of the samples with higher variations in the Eastern Mediterranean region was clearly observed. Moreover, remarkable variations were recognized among *Q. coccifera* populations.

Research highlights: The samples from region called Levant quite possibly strengthen the presence of two different species as *Q. coccifera* and *Q. calliprinos* or two intraspecific taxa such as *Q. coccifera* subsp *coccifera* and *Q. coccifera* subsp *calliprinos*. However, this needs to be supported by studies including *Q. calliprinos* and *Q. coccifera* samples from East Mediterranean region of Türkiye to Egypt.

Keywords: *Q. coccifera*, *Q. calliprinos*, matK Gene-partial trnK Gene Intron, rbcL Gene

Q. coccifera L. İçerisindeki Genetik Varyasyonların Analizi ve Populasyonlar Arasındaki İlişkilerde Coğrafik Farklılıkların Etkisi

Öz

Çalışmanın amacı: Bu çalışma, *Q. coccifera* içerisindeki varyasyonları ve populasyonlar arasındaki varyasyonlar üzerine coğrafik farklılıkların etkisini belirlemeyi amaçlamaktadır.

Çalışma alanı: Akdeniz ülkelerinin *Q. coccifera* populasyonlarını içeren bu çalışmaya ait örnekler, NCBI veri tabanından temin edilmiştir.

Materyal ve yöntem: Bu çalışmada, matK geni-kısmi trnK gen intronu ve rbcL gen sekansları temelinde, *Q. coccifera* ve *Q. calliprinos*'a ait tüm populasyonlar, NCBI veri tabanından toplandı. Popülasyonların ilişkilerini ve varyasyonlarını ortaya çıkarma yetenekleri açısından barkodlama bölgelerini değerlendirmek ve popülasyonların filogenetik ilişkileri hakkında daha bilgilendirici sonuçları belirlemek amaçlandı. Moleküler Evrimsel Genetik Analiz (MEGA11) bu amaçla kullanıldı.

Temel sonuçlar: Barkodlama bölgelerinin her ikisi için, Doğu Akdeniz bölgesinde daha yüksek varyasyonlu örneklerin varlığı açık bir şekilde gözlenmiştir. Bununla birlikte *Q. coccifera* populasyonları arasında dikkat çekici varyasyonlar ayırt edildi.

Araştırma vurguları: Levant olarak adlandırılan bölgeden örnekler, oldukça olası bir şekilde *Q. coccifera* ve *Q. calliprinos* olarak iki farklı türün veya *Q. coccifera* subsp *coccifera* ve *Q. coccifera* subsp *calliprinos* olarak iki tür içi taksonun varlığını güçlendirmektedir. Ancak, bu durumun Türkiye'nin Doğu Akdeniz bölgesinden Mısır'a, *Q. calliprinos* ve *Q. coccifera* örnekleri içeren çalışmalarla desteklenmesi gerekmektedir.

Anahtar kelimeler: *Q. coccifera*, *Q. calliprinos*, matK Geni-partial trnK Gen İntronu, rbcL Geni

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Introduction

Using of DNA regions containing sufficient sequence variations is an important approach in the phylogenetic and evolutionary studies. Especially, the species identification of closely related taxa and the grouping of species in terms of common characteristics is very hard and important in taxonomically problematic plant groups (Yılmaz, 2020a). For this reason, based on their ability to reveal the relationships, the choice of suitable barcoding sequences is the most valuable and favourable attempt especially in the problematic plant groups. However, same barcoding regions may show the variations according to their species identification and separation abilities in different plant groups. This makes it necessary to have knowledge about the ability of different barcoding regions for plant groups in order to solve taxonomic problems and to evaluate phylogenetic relationships. As a result of this, it can be stated that it will provide more accurate and meaningful results for further studies consisting of combinations of correct barcoding regions.

The genus *Quercus* L. is a problematic plant group with over 500 woody plant species distributed in the northern hemisphere (Kremer & Petit, 1993; Manos et al., 2001; Borazan & Babaç, 2003; Hubert et al., 2014; Yılmaz, 2018a; 2018b; Yılmaz, 2020a; Ngoc et al., 2022). There are many situations that increase the taxonomic problems in the genus such as hybridization, introgression, and wide geographical distribution. Oaks are the widespread and outcrossing species because of wind pollination and weak reproductive barriers (Hokanson et al., 1993; Kremer & Petit, 1993; Arnold, 1997; Rieseberg & Willis, 2007; Neophytou et al., 2010; Yılmaz et al., 2013; Yılmaz, 2018b). Most oak species grow in mixed populations that exhibit hybridization behaviour especially within the same section or group in different geographical regions (Bacilieri et al., 1996; Petit et al., 2004; Charalambos et al., 2011; Yılmaz, 2016). Wide geographical distribution of the *Quercus* species in the habitats that have different ecological and climatic conditions bring about different gene flow mechanism and hybrid individuals that exhibit intermediate morphological characters between parent taxa (Denk & Grimm, 2010; Simeone et al., 2013; Yılmaz, 2017). All these lead to mistakes in the species identification and finally increase the taxonomic problems. Genetic drift, epigenetic mechanisms, the lack of conservation programs (Öztürk & Özdemir, 2013) in adequate level–especially for some species that have high economic value and hence used for many purposes are the other important reasons that effect the right evaluation of the genus taxonomically and phylogenetically.

Many studies based on molecular, morphological and cytogenetics have been conducted to determine and later solve taxonomic problems, besides understanding the phylogenetic relationships within the genus *Quercus*. DNA barcoding studies that contain short sequence information belonging to nuclear and chloroplast DNA (cpDNA) have been widely used recently (Denk & Grimm, 2010; Piredda et al., 2011; Yılmaz, 2020a; 2020b; Ngoc et al., 2022). Especially, many regions belonging to chloroplast genome that contain both of gene and spacer sequence information have been commonly used in multiple plant groups. Nevertheless, uncertainty in the taxonomic and systematic relationships of the genus *Quercus* have not still been eliminated. It is vital to determine the DNA sequences that have the best discrimination ability within the barcoding regions that show variation in terms of their species identification abilities in plant groups, and to use their combinations for the best evaluation in taxonomically and phylogenetically problematic genera.

The distinction between *Q. coccifera* and *Q. calliprinos* Webb belonging to the genus *Quercus* is not very clear and they are still confused with each other (Toumi & Lumaret, 2001; Yılmaz et al., 2017). Furthermore, the evaluation of these two taxa by many researchers show differences (Salvatore & Paola, 1976; Toumi & Lumaret, 2001; Yılmaz et al., 2017). In other words, it is still controversial whether *Q. coccifera* and *Q. calliprinos* are separate species or subspecies within *Q. coccifera* as *Q. coccifera* subsp. *coccifera* and *Q. coccifera* subsp. *calliprinos*. Similarly, Vila-Viçosa et al. (2023) states in their study based on Portuguese oaks that Kermes oaks (*Q. coccifera*) are immersed in

nomenclatural controversy. *Q. calliprinos* is widely used to adress the eastern Mediterranean kermes oaks, while the original taxon is described from Northwest Africa referring to a tree with tomentose leaves (Vázquez et al., 2018; Vila-Viçosa et. al. 2022). The most important and distinctive character between *Q. coccifera* and *Q. calliprinos* is downy leaves from both faces that led Webb (1838) to propose a new species (Vila-Viçosa et al., 2023). Moreover, *Q. aucheri* was evaluated as proximate of this taxon by Candolle (1864), even as a putative hybrid between *Q. ilex* and *Q. calliprinos* by Kasapligil (1981). As a result, it can be said that taxonomic status of these taxa are still controversial.

It is stated that the living area of *Q. calliprinos* populations is East Mediterranean region and shows difference with *Q. coccifera* in terms of distribution areas (Toumi & Lumaret, 2001). In this concept, variations phylogenetically for the populations provided from East Mediterranean region that consist of Lebanon, Israel, Jordan and Türkiye, in addition to adjacent Island populations of Greece is expected as a result of this study.

In this study, all populations belonging to *Q. coccifera* with together *Q. calliprinos* evaluated in species or subspecies level based on matK gene-partial trnK gene intron and rbcL gene sequences provided from National Center for Biotechnology Information (NCBI) were collected and later it was aimed to ***i)*** evaluate the barcoding regions examined in terms of their ability to reveal the relationships between populations belonging to different localities ***ii)*** present more comprehensive and informative results about the taxonomic and phylogenetic relations of taxa examined, and finally ***iii)*** make suggestions about taxonomic status of some *Q. coccifera* populations according to the results provided from phylogenetic tree.

Material and Methods

All sequence data of matK gene-partial trnK gene intron and rbcL gene belonging to cpDNA were acquired from NCBI database and subsequently analysed based on their sequence compatibilities. Compatible sequences were detected for the two regions containing matK and rbcL gene and these were examined in the study for further analysis. In this study, with the sequence selection by the criteria such as the sequence sharing by different researchers at different periods in NCBI, the use of as many samples belonging to *Q. coccifera* as possible, it was aimed

-to reveal the most comprehensive relationships among *Q. coccifera* populations belonging to different localities

-to determine the populations that show the highest variation within the *Q. coccifera*

-to uncover the possible populations of *Q. calliprinos* whose taxonomic status is uncertain.

Firstly, sequence alignments for both regions of interest were performed by using Molecular Evolutionary Genetics Analysis (MEGA 11) (Tamura et al., 2021). 28 populations from 16 countries for matK gene-partial trnK gene intron and 26 populations from 16 countries for rbcL gene were examined based on sequence information. GenBank codes are given in Supplementary Material Table S1.

Variable and parsim-info sites are important indicators for identifying and separating species phylogenetically for the barcoding regions examined. Nucleotide sequences were computed for both and variable sequences for each barcoding region were shown in Supplementary Material Table S2 and S3. The probabilities of base substitutions, transitional base substitutions in addition to transversional base substitutions (%), transition/transversion ratios for purines, pyrimidines and overall, nucleotide frequencies as A+T/U % and G+C % were computed for both barcoding regions. Finally, the Maximum Parsimony (MP) method which infer the evolutionary history was performed to show the phylogenetic relationships of populations from different localities and to determine the populations which have highest variations. The positions with gaps and missing data in the sequences alignment were eliminated using the program's complete deletion option for more effective analyses.

Results

All sequence information for matK gene-partial trnK gene intron and rbcL gene acquired from the NCBI database were made

compatible to obtain significative results for *Q. coccifera* populations. The 28 populations for the region containing matK sequences and 26 populations for rbcL gene sequences were determined and used for further analysis. The alignment lengths of the populations for both matK gene-partial trnK gene intron and rbcL sequences were determined as 694 bp and 743 bp, respectively. The variable nucleotide sequences and parsimony informative sequences were determined in 12 and 9 sites for matK gene-partial trnK gene intron, respectively, while they were observed in 7 and 5 nucleotides for rbcL gene. It can be stated for both regions examined that the substitutions between nucleotides for variable and parsim-info sites are observed in only a few nucleotides. However, important variations in the variable nucleotide sequences which have crucial data in species separation and their phylogenetic relationships were recognized among *Q. coccifera* populations belonging to different habitats (Supplementary Material Table S2 and S3). The probabilities of substitutions between bases were determined and the highest substitutions were detected as 29.31% from C to T and 13.09% from T to C, for the region containing matK gene-partial trnK gene intron. Furthermore, transitional substitutions and transversional substitutions were computed using Table 1 as 47.71% and 52.29%, respectively. Additionally, it can be stated that transversional substitutions are higher than the transitional substitutions according to the results provided from matK gene-partial trnK gene intron.

Table 1. The substitution probabilities of bases for matK gene-partial trnK gene intron (Transitional substitutions are shown in bold)

	A	**T**	**C**	**G**
A	-	8.96	4	**1.71**
T	8.95	-	**13.09**	4.24
C	8.95	**29.31**	-	4.24
G	**3.6**	8.96	4	-

When the substitution probabilities from a base to another one were investigated, the highest substitutions were observed as 19.11% from C to T and 14.51% from T to C, for the region containing rbcL gene sequences (Table 2).

Transitional substitutions and transversional substitutions were computed as 54.77% and 45.23%, respectively. In other words, it can be stated that transitional substitutions in comparison to the transversional substitutions are higher for rbcL gene sequences, contrary to the results provided from matK gene-partial trnK gene intron.

Table 2. The substitution probabilities of bases for rbcL gene (Transitional substitutions are shown in bold)

	A	**T**	**C**	**G**
A	-	6.31	4.79	**9.46**
T	6.36	-	**14.51**	5.15
C	6.36	**19.11**	-	5.15
G	**11.69**	6.31	4.79	-

In the comparison of transition/transversion ratio of purines (k_1) and pyrimidines (k_2) for matK gene-partial trnK gene intron, pyrimidines with 3.27 show higher value than purines. Overall transition/transversion ratio (R) that includes all positions in the final dataset was determined as 0.77 (Table 3). The transition/transversion ratio for purines (k_1), pyrimidines (k_2) and also overall transition/transversion ratio were determined as 1.83, 3.02 and 1.18, respectively for rbcL gene. In other words, pyrimidines according to the transition/transversion ratio show higher value than purines in the comparison, similar to the results provided from matK gene-partial trnK gene intron (Table 3).

Nucleotide frequencies for the sequences belonging to matK gene-partial trnK gene intron and rbcL gene were analysed, and the percentage of A+T/U bases in addition to G+C bases was computed as 68.48 and 31.52 for matK gene-partial trnK gene intron, 56.04 and 43.96 for rbcL gene sequences, respectively. It can be stated for both barcoding regions that DNA sequences analysed for *Q. coccifera* populations consist of highly A and T/U bases (Table 3).

Table 3. The information of the all populations based on barcoding sequences examined

DNA regions	Popul. numb.	Align. length (bp)	Var. site	Parsim -info site	Transit. substit. (%)	Transv. substit. (%)	Transit./Transvers. Purine (k_1)	Pyrimid. (k_2)	Overall (R)	Nucleotide freq. (%) A+T/U	G+C
matK-trnK intr.	28	694	12	9	47.71	52.29	0.40	3.27	0.77	68.48	31.52
rbcL gene	26	743	7	5	54.77	45.23	1.83	3.02	1.18	56.04	43.96

Finally, Maximum Parsimony (MP) dendrograms which are effective in evaluating taxa in terms of their phylogenetic relationships and taxonomic statues were drawn for each barcoding regions (Figure 1, 2). The dendrogram provided from matK gene-partial trnK gene intron separated the populations belonging to *Q. coccifera* and *Q. coccifera* var. *calliprinos* into four main groups. It can be stated that the populations from localities that have different ecological and climatic conditions were clustered according to their geographical distribution (Figure 1). The other dendrogram provided from rbcL gene sequences separated the *Q. coccifera* populations from different localities into three groups. Although the clustering of the populations examined shows similarity with the dendrogram provided from matK gene-partial trnK gene intron, it is observed that the clustering of the populations in terms of geography is not as clear as the other one (Figure 2).

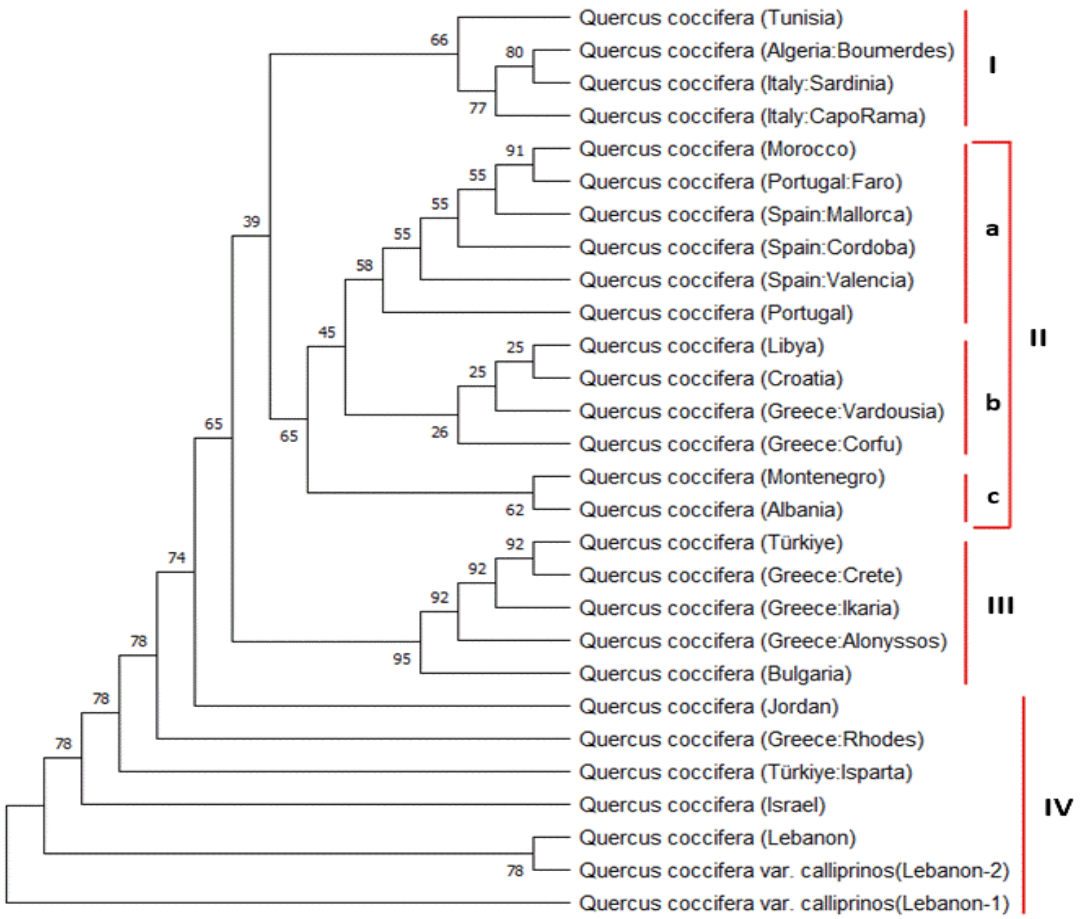

Figure 1. Maximum Parsimony tree provided from matK gene-partial trnK gene intron

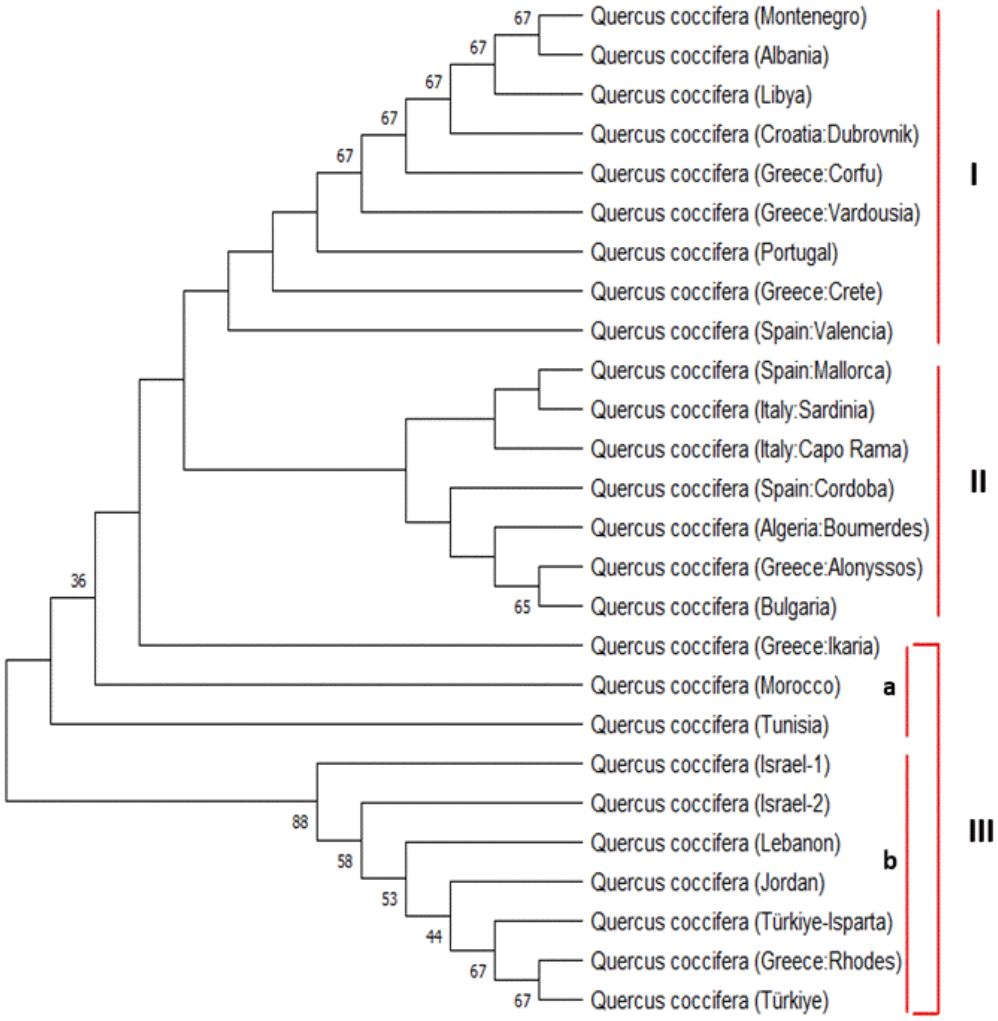

Figure 2. Maximum Parsimony tree provided from rbcL gene

Discussion

Variations in morphological characters make it difficult for researchers to identify species correctly. Furthermore, the plant samples displaying intermediate morphological characters caused by hybridization are another reason that lead the taxonomic problems and misclassifications. Wide geographical distribution affecting the species with different climatic and ecological factors, hybridization observed between species with weak reproductive barriers, destruction of distribution areas for some species of high economic value, inadequate conservation programs and the lack of comprehensive studies are the main reasons that increase the variations and taxonomic problems in plants. All of them are the situations observed in the genus *Quercus*. *Q. coccifera*, which has a wide geographical distribution and diversity with the populations belonging to different habitats is the problematic species in the genus. The utilization of short DNA sequences to reveal variations and phylogenetic relationships of taxa is an important approach in the evaluation of species such as *Q. coccifera* that show variations according to their distribution area.

Phylogenetic tree provided from matK gene-partial trnK gene intron separated the populations examined into four main groups. Group I consists of the samples collected in Algeria (Boumerdes), Tunisia, two populations of Italy (Sardinia and Capo Rama) (Figure 1). It can be stated that all populations belonging to *Q. coccifera* are very close in terms of their localities. Furthermore, Sardinia and Capo Rama are the populations from Italy that have the closest localities to

Algeria (Boumerdes) and Tunisia. The populations in group II showed three separate clustering as a, b, and c. Cluster a consists of the populations from Morocco, Portugal, Spain, and these are adjacent populations. Cluster b and c consist of the populations belonging to Montenegro, Albania, Croatia, Greece (Corfu and Vardouisa), and Libya. Thus, it can be stated that the populations examined have considerable close localities. Island populations of Greece in the Aegean Sea and adjacent areas here, along with *Q. coccifera* populations from Türkiye and Bulgaria, generate group III that consists of outmost populations in dendrogram after group IV. Group IV populations formed outmost clade in MP tree. *Q. coccifera* populations represented by Group IV consist of an island population of Greece (Rhodes) in between the Aegean Sea and the coasts of the Middle East, Isparta population from Southwest Türkiye, Jordan, Israel, and Lebanon populations. Two populations belonging to *Q. coccifera* var. *calliprinos* merged from outermost to this group and formed the samples that have highest variations.

In the MP tree of matK gene-partial trnK gene intron (Figure 1), it is observed that the populations showing the highest variation according to their sequence information provided from NCBI database belong to *Q. coccifera* var. *calliprinos* and then *Q. coccifera* that have close distribution to this area.

Moreover, it can be stated that *Q. coccifera* populations collected from different regions were clustered based on their geographical distribution. In the study based on molecular diversity of Turkish oaks, Yılmaz et al. (2013) in the study on oaks using molecular approach determined the highest variations in the populations of *Q. coccifera* distributed in the East Mediterranean region. A similar result was observed in the study of Yılmaz et al. (2017) based on the morphological variability of oaks. In other words, the regions geographically closer to Syria, Lebanon, Jordan, and Israel show highest variations within *Q. coccifera* populations. Furthermore, as a result of both studies it was stated that two groups showing geographical differences within *Q. coccifera* reinforce the presence of two species as *Q. coccifera* and *Q. calliprinos* or two different taxa at species level as *Q. coccifera* subsp. *coccifera* and *Q. coccifera* subsp. *calliprinos* (Yılmaz et al. 2013; Yılmaz et al. 2017). Zohary (1966) states the presence of *Q. calliprinos* species, also two subspecies of *Q. calliprinos* as *Q. calliprinos* subsp. *coccifera* and *Q. calliprinos* subsp. *calliprinos* in Flora of Palestine. In their study based on climatic adaptations of the *Q. coccifera* samples, Ozturk & Altay (2021) state that ‘‘In the Levant, traditionally *Q. coccifera* is named *Q. calliprinos* (Palestine oak) based on the morphological characters’’. However, there is no consensus on whether to classify Eastern Mediterranean taxon as a distinct species or subspecies (Blondel & Aronson, 1999; Tutin et al., 2010; Ozturk & Altay, 2021).

Phylogenetic tree provided from rbcL sequences separated the populations examined into three main groups.

The highest variations are observed in the populations belonging to Israel, Lebanon, Jordan, Türkiye (Isparta) and Greece (Rhodes). As a result of the dendrogram provided from rbcL sequences of the populations belonging to the *Q. coccifera*, it can be stated that similar clustering is observed based on geographical distribution. However, phylogenetic relationships are not as clear as data provided from matK gene-partial trnK gene intron (Figure 2).

In the study that aims to better understand the phylogeography and evolution of the Lebanon oaks, Douaihy et al. (2020) state that Levant region is an important biogeographical crossroad between continents in diversification and hence the presence of the important areas in Lebanon in this concept. The dendrograms provided from both of the DNA barcoding regions show that populations that have distribution areas in these regions exhibit the highest variations within *Q. coccifera*. Similarly, Yılmaz et al. (2013; 2017) state that the highest variations within *Q. coccifera* were observed in populations collected from East Mediterranean region such as Gaziantep, Kahramanmaraş and Hatay. As a result, they suggested that the variations within *Q. coccifera* quite possibly strengthen the presence of two different species as *Q. coccifera* and *Q. calliprinos* or two

intraspecific taxa such as *Q. coccifera* subsp *coccifera* and *Q. coccifera* subsp *calliprinos*. However, this needs to be supported by studies including *Q. coccifera* samples from East Mediterranean region of Türkiye to Egypt.

Conclusion

Considering the sequence information of matK gene-partial trnK gene intron and rbcL gene, it can be clearly stated that there is a second group with higher variations than others within *Q coccifera* and belongs it to the Eastern Mediterranean region. These variations observed in *Q. coccifera* populations belonging to region called Levant were evaluated by many researchers and showed differences taxonomically. Yılmaz et al. (2013; 2017) in their both studies based on molecular diversity and morphological variability of oaks determined the highest variations within the *Q. coccifera* in the regions geographically closer to Levant. Furthermore, they stated that the variations within *Q. coccifera* reinforce the presence of two species as *Q. coccifera* and *Q. calliprinos* or two different taxa as *Q. coccifera* subsp. *coccifera* and *Q. coccifera* subsp. *calliprinos*. Zohary (1966) and Ozturk & Altay (2021) state the presence of *Q. calliprinos* species in the Levant. Similarly, Douaihy et al. (2020) in their study on the Lebanon oaks state that Levant is an important biogeographical region in diversification. In this concept, this study based on cpDNA sequences is very important to reveal the most comprehensive relationships among *Q. coccifera* populations.

However, the problems related to the data sharing in NCBI, such as country information of taxa not uploaded to the database, in addition to unspecified habitats of taxa studied from the countries distributed to a wide geographical area with different ecological and climatic conditions, make it difficult to determine the variations within the species and then to interpret them. Türkiye is one of the countries distributed to wide geographical regions, and it has been under the influence of numerous phytogeographic regions such as Irano-Turanian, Euro-Siberian, and Mediterranean. This increases the importance of habitats in the evaluation of oak variations, especially for *Q. coccifera* distributed along coastal regions of the Mediterranean Sea.

Although both barcoding regions belonging to the chloroplast genome support that the populations from the Eastern Mediterranean region show the highest variation, the region containing matK sequences is particularly recommended for its ability to reveal the phylogenetic relationships among the populations in a more detailed, clear, and meaningful way.

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N/A

Peer-review

Externally peer-reviewed.

Author Contributions

Conceptualization: A.Y.; Investigation: A.Y.; Material and Methodology: A.Y., O.D., R.T.; Visualization: A.Y.; Writing-Original Draft: A.Y., O.D., R.T.; Writing-review & Editing: A.Y., O.D., R.T. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

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