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Analysis of the Variations Within *Quercus ilex* L. and the Evaluation of Morphological Types Based on Chloroplast and Nuclear DNA Sequences

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ABSTRACT

Quercus ilex, evaluated within evergreen oaks, has a wide geographic distribution in the Mediterranean basin. Hybridization and gene flow are effective and frequently observed mechanisms in *Q. ilex*. Additionally, weak reproductive barriers between closely related taxa in zones of geographical contact further increase genetic diversity and subsequent taxonomic problems. Two morphological types, known as rotundifolia and ilex, are defined based on the variations between *Q. ilex* populations appearing as a result of all these factors. However, it is still controversial whether morphological types: ilex and rotundifolia are subspecies of *Q. ilex* or two separate species. In this study, short DNA sequences that consist of matK gene-partial trnK gene intron of chloroplast DNA and ITS1-5.8S rRNA gene-ITS2 of nuclear DNA were used to overcome such difficulties and to reveal the variations between *Q. ilex* populations. All *Q. ilex* populations based on both barcoding regions were determined and examined using the Molecular Evolutionary Genetics Analysis (MEGA 11). The analysis such as base substitutions, variable and parsim-info sites, transitional and transversional base substitution ranges (%), and nucleotide frequencies (%) was performed and transitional substitutions according to the transversional substitutions for both barcoding regions were observed in the high-value. Furthermore, the sequences belonging to nuclear DNA in comparison to other barcoding regions exhibited higher variable and parsim-info sites. Finally, Maximum Parsimony (MP) dendrograms for both barcoding regions were drawn to evaluate the populations belonging to *Q. ilex* in terms of their variations, phylogenetic-evolutionary relationships, and taxonomic status. Although both barcoding regions support the separation of *Q. ilex* populations based on different morphological types, matK gene-partial trnK gene intron sequences exhibited clearer and more informative results than ITS1-5.8S rRNA gene-ITS2 sequences.

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Quercus ilex L. İçerisindeki Varyasyonlarin Analizi ve Kloroplast ve Nükleer DNA Sekanslari Temelinde Morfolojik Tiplerin Değerlendirmesi

ÖZET

Herdem yeşil meşeler içerisinde değerlendirilen *Quercus ilex*, Akdeniz temelinde geniş coğrafik dağılıma sahiptir. Hibridizasyon ve gen akışı *Q. ilex*'de etkili ve sıklıkla gözlenen mekanizmalardır. Ayrıca, coğrafik olarak temaslı bölgelerde, yakın ilişkili taksonlar arasında zayıf üreme bariyerleri, *Q. ilex* içerisindeki genetik çeşitliliği ve sonrasında taksonomik problemleri arttıran diğer bir önemli durumdur. Rotundifolia ve ilex olarak bilinen iki morfolojik tip, tüm bu faktörlerin sonucu olarak ortaya çıkan, *Q. ilex* populasyonları arasındaki varyasyonlar temelinde tanımlanır. Ancak, morfolojik tipler: ilex ve rotundifolia nın *Q. ilex*'in alttürlerimi yoksa iki ayrı türmü olup olmadığı hala tartışmalı durumdur. Bu çalışmada, kloroplast DNA'ya ait matK geni-kısmi trnK gen intronu ve nükleer DNA'ya ait ITS1-5.8S rRNA geni-ITS2 den oluşan kısa DNA

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sekansları, bu tarz zorlukların üstesinden gelmek ve *Q. ilex* populasyonları arasındaki varyasyonları ortaya çıkarmak için kullanıldı. *Q. ilex*'e ait tüm populasyonlar heriki barkodlama bölgesi temelinde belirlendi ve Molecular Evolutionary Genetics Analysis (MEGA 11) kullanarak incelendi. Baz değişimleri, varyasyonlu ve parsim info bölgeler, transisyonel ve transversiyonel baz değişim oranları (%) ve nükleotid frekansları (%) gibi analizler gerçekleştirildi ve her iki barkodlama bölgesi için transisyonel baz değişimlerinin transversiyonel değişimlere göre daha yüksek değerde olduğu gözlemlendi. Ayrıca, nükleer DNA'ya ait sekanslar diğer barkodlama bölgesi ile karşılaştırmada daha yüksek varyasyonlu ve parsim info bölgeler sergiledi. Son olarak her iki barkodlama bölgesi için Maximum Parsimony (MP) dendrogramlar, varyasyonlar, filogenetik-evrimsel ilişkiler ve taksonomik statüler açısından *Q. ilex*'e ait populasyonları değerlendirmek için çizildi. Her iki barkodlama bölgesi, *Q. ilex* populasyonlarının farklı morfolojik tipler temelinde ayrımını desteklemesine rağmen, özellikle matK geni-kısmi trnK gen intron sekansları, ITS1-5.8S rRNA geni-ITS2 sekanslarından daha açık ve bilgilendirici sonuçlar sergiledi.

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INTRODUCTION

Quercus ilex L., commonly known as holm oak, is an evergreen tree or shrub a natural distribution across the central and western Mediterranean basin, Aegean Islands, Balkan regions, North Africa, western parts of Türkiye and the limited coastal areas of Black Sea in Türkiye (Barbero et al., 1992; de Rigo & Caudullo, 2016; Suicmez & Avci, 2023).

Q. ilex, a dominant species in the Mediterranean forests, faces threats from various factors such as vertebrate and invertebrate species that rely on this tree for sustenance and habitat, differentiation in the geographical distribution under ecological and climatic changes, destruction for its high economic value and inadequate protection strategies (Yılmaz, 2018; Hernandez-Agüero et al., 2022; Rey et al., 2023; Suicmez & Avci, 2023). Additionally, the aging tree populations with poor regeneration capacity also impact species diversity and distribution (Rey et al., 2023).

Q. ilex, evaluated within evergreen oaks, has two main morphological types known as rotundifolia and ilex (Saenz de Rivas, 1967, 1970; Peguero-Pina et al., 2014; Bensaci et al., 2021). The rorundifolia type has small and round thick leaves, while the ilex type features elongated and large pointed leaves (Tutın et al., 1964; Peguero-Pina et al., 2014). Furthermore, three different morphotypes - ilex, rotundifolia, and intermediate - within holm oak were defined by Michaud et al. (1995) and Lumaret et al. (2002). The distribution areas for the individuals with intermediate morphotypes exhibiting characteristics between ilex and rotundifolia were identified in coastal areas of eastern and northern Spain and south France (Languedoc and Roussillon). Rotundifolia morphotype is characterized by dry distribution areas of the Mediterranean climate such as North Africa and the interior region of Spain (Tutın et al., 1964; Lumaret et al., 2002; Vázquez Pardo et al., 2002; Peguero-Pina et al., 2014). Ilex morphotypes are distributed from Greece to the French Riviera along France's Atlantic coast (Lumaret et al., 2002; Peguero-Pina et al., 2014). In addition to the distribution areas stated for morphotypes, *Q. ilex* has natural populations in Türkiye. Yılmaz et al. (2013) evaluated the *Q. ilex* in five separate populations in their study based on the molecular diversity of evergreen oaks in Türkiye. Similarly, the relationships of *Q. ilex* populations based on their morphological variabilities were examined by Yılmaz et al. (2017). Comprehensive studies, including *Q. ilex* populations in Türkiye, are necessary to better understand the species' genetic diversity.

The classification of morphological types (ilex and rotundifolia) within *Q. ilex* as either subspecies (*Q. ilex* subsp. *ilex* and *Q. ilex* subsp. *rotundifolia*) or separate species remains controversial (Tutın et al., 1964; Saenz de Rivas, 1967; Amaral-Franco, 1990; Govaerts and Frodin, 1998; Vázquez Pardo et al., 2002; Soto et al., 2007; de Rigo & Caudullo, 2016; Sousa et al., 2021).

Furthermore, the taxonomic status of these morphotypes has not been completely clear and resolved yet. Today,

the separation of ilex and rotundifolia types within *Q. ilex* is based on their morphological characters and geographic distribution.

Hybridization and gene flow are effective mechanisms frequently observed in the genus *Quercus*. They are important processes in genetic diversity, evolution, and speciation of the genus. Hybridization, commonly observed between taxa with weak reproductive barriers in zones of geographical contact, complicates taxonomy (Bacilieri et al., 1996; Borazan & Babaç; 2003). Similar hybridization behaviors in *Q. ilex* were reported by Schnitzler et al. (2004) and Lopez de Heredia et al. (2018). *Quercus* x *turneri* 'Pseudoturneri' as a hybrid resulting from a crossing of *Q. ilex* L. and *Q. robur* L. is stated by Schnitzler et al. (2004). Hybridization between *Q. suber* L. and *Q. ilex* L. in the zones where they form mixed stands has been known and reported for a long time (Lopez de Heredia et al., 2018). Two evergreen oaks: *Q. ilex* and *Q. coccifera* are closely related taxa that have extensive distribution areas in the Mediterranean region and co-occurred in mixed stands where hybridization may take place (de Casas et al. 2007; Ortego & Bonal, 2010). Consequently, introgression as a result of hybridization between *Q. ilex* and *Q. coccifera* in the distribution areas overlapping frequently appeared (Jimenez et al., 2004; Lopez de Heredia et al., 2007; Ortego & Bonal, 2010).

These factors have an important effect on the variation in morphological characters and distribution of *Q. ilex*. Variations within oak species complicate species identification due to fuzzy species boundaries. (Bacilieri et al., 1996; Borazan & Babaç; 2003; Petit et al., 2003; Yılmaz, 2018). All these factors increase the taxonomic problems in *Q. ilex* which has extensive distribution areas and makes problematic the taxon. To overcome such difficulties and to collect the taxa in the correct systematic categories, short DNA sequences that contain enough information to identify the species and reveal the phylogenetic relationships between taxa are frequently used as a molecular approach. In this study, matK gene-partial trnK gene intron sequence data of chloroplast DNA and ITS1-5.8S rRNA gene-ITS2 sequence data of nuclear DNA were acquired from the National Center for Biotechnology Information (NCBI) and later it was aimed to: i) evaluate the ability of chloroplast and nuclear DNA barcoding regions to reveal phylogenetic relationships among *Q. ilex* populations from different localities, ii) determine the variations between *Q. ilex* populations, iii) create a phylogenetic tree and make suggestions about the taxonomic status of *Q. ilex* populations according to the results provided from the phylogenetic tree, and iv) present more informative and comprehensive results about the taxonomic and phylogenetic relations of morphological types within *Q. ilex*.

MATERIALS and METHODS

All sequence data for *Q. ilex* populations, covering both barcoding regions (matK gene-partial trnK gene intron of chloroplast DNA and ITS1-5.8S rRNA gene-ITS2 sequence of nuclear DNA) from past to present, were obtained from the NCBI database. Sequences of the 18S rRNA gene- ITS1- 5,8S rRNA gene- ITS2- 28S rRNA gene, in addition to ITS1-5.8S rRNA gene-ITS2, were collected and then the sequences containing ITS1- 5,8S rRNA gene-ITS2 were extracted from these regions. Finally, sequence data of all extracted regions for *Q. ilex* populations were combined to provide more effective and comprehensive results about the variations between *Q. ilex* populations and the taxonomic status of morphological types within *Q. ilex*.

A total of 37 *Q. ilex* populations were analyzed for compatibility of sequence information based on matK gene-partial trnK gene intron. Additionally, 20 populations of *Q. ilex* for the region containing ITS1 and ITS2 sequence data were detected and examined in this study. GenBank codes for both barcoding regions were presented in the Appendix. The multiple sequence alignments for *Q. ilex* populations were separately performed for both DNA sequences using the Molecular Evolutionary Genetics Analysis (MEGA 11) (Tamura et al. 2021). The probabilities of substitution from one base to another base were determined and subsequently, variable and parsim-info sites which are important indicators in phylogenetic relationships were computed for both barcoding regions belonging to nuclear and chloroplast DNA. Transitional and transversional base substitution ranges (%) were computed for examined DNA sequences. Finally, nucleotide frequencies of matK gene-partial trnK gene intron and ITS1-5.8S rRNA gene-ITS2 sequences were determined and presented as G+C % and A+T/U %.

Dendrograms showing bootstrap values on their branches and inferred the evolutionary history were created using the Maximum Parsimony (MP) method. These MP dendrograms for both barcoding regions were used to evaluate the phylogenetic relationships between *Q. ilex* populations, determine the variations among populations from different geographic regions, and provide more informative results about morphological types within *Q. ilex*.

RESULTS and DISCUSSION

The sequence data for ITS1-5.8S rRNA gene-ITS2 of nuclear DNA and, matK gene-partial trnK gene intron of chloroplast DNA were acquired from the NCBI for all *Q. ilex* populations. The alignment lengths for a total of 37 *Q. ilex* populations were determined as 695 bp based on the sequence information of matK gene-partial trnK gene

intron. The variable and parsimony informative sites expressing the nucleotide substitutions are critical indicators for determining the variations and relationships among morphological types in *Q. ilex* populations from different habitats. In this study, based on the matK gene-partial trnK gene intron, the variable, and parsimony informative sites were observed in 9 and 8 nucleotides, respectively. The probabilities of substitutions between bases for matK gene-partial trnK gene intron were determined and shown in Table 1.

Table 1. The probabilities of substitution (r) from one base (row) to another base (column) for matK gene-partial trnK gene intron (Transitional substitutions are shown in bold).

Çizelge 1. matK geni-kısmi trnK gen intronu için bir bazdan diğerine değişim olasılıkları (Transisyonel baz değişimleri koyu renkli gösterilir)

	A	**T**	**C**	**G**
A	-	*6.9*	3.06	**0.58**
T	6.71	-	**17.89**	3.32
C	6.71	**40.37**	-	3.32
G	**1.17**	6.9	3.06	-

The highest substitutions were observed as 40.37% from C to T and then 17.89% from T to C. Moreover, transitional and transversional base substitutions were computed from Table 1 as 60.01% and 39.99%, respectively. This indicates that transitional substitutions are higher than the transversional substitutions for matK gene-partial trnK gene intron sequences belonging to *Q. ilex* populations.

The alignment lengths for a total of 20 *Q. ilex* populations based on the ITS1-5.8S rRNA gene-ITS2 sequences were determined to be 604 bp. The variable and parsimony informative sites were observed in 58 and 32 nucleotides, respectively. The probabilities of substitutions between bases for ITS1-5.8S rRNA gene-ITS2 sequences were determined and the highest substitutions detected as 33.02% from T to C and then 20.24% from A to G. (Table 2). Moreover, transitional and transversional base substitutions were computed as 83.81% and 16.19%, respectively. This indicates that transitional substitutions are significantly higher than the transversional substitutions for the region containing ITS1-5.8S rRNA gene-ITS2 sequences.

Table 2. The probabilities of substitution (r) from one base (row) to another base (column) for ITS1-5.8S rRNA gene-ITS2 sequences (Transitional substitutions are shown in bold).

Çizelge 2. ITS1-5.8S rRNA geni-ITS2 sekansları *için bir bazdan diğerine değişim olasılıkları (Transisyonel baz değişimleri koyu renkli gösterilir)*

	A	**T**	**C**	**G**
A	-	*1.45*	2.69	**20.24**
T	1.52	-	**33.02**	2.43
C	1.52	**17.85**	-	2.43
G	**12.7**	1.45	2.69	-

Transition/transversion ratios for purines (k_1) and pyrimidines (k_2) were determined and compared for both barcoding regions. It was observed that the transition/transversion ratio of pyrimidines (5.84) was higher than that of purines for matK gene-partial trnK gene intron sequences. The transition/transversion ratio for purines (k_1), pyrimidines (k_2), and overall were determined as 8.34, 12.27, and 4.81, respectively for ITS1-5.8S rRNA gene-ITS2 sequences. In other words, pyrimidines in the aspect of the transition/transversion ratio show a higher value than purines in the comparison, similar to the results provided from matK gene-partial trnK gene intron (Table 3).

It was determined that DNA sequences analyzed for *Q. ilex* populations consist primarily of A and T/U bases (68.09%) for the region that contains matK gene-partial trnK gene intron sequences. Conversely, it was observed that the percentage of G and C bases was higher (63.22%) than A+T/U bases (36.78%) for ITS1-5.8S rRNA gene-ITS2 sequences (Table 3).

Furthermore, table 4 shows the variable nucleotides for both barcoding regions were formed to understand the phylogenetic and evolutionary relationships between *Q. ilex* populations.

Finally, Maximum Parsimony (MP) dendrograms for both barcoding regions were drawn to evaluate the *Q. ilex* populations in terms of their variations, phylogenetic-evolutionary relationships, and taxonomic status (Figure 1, 2).

The examined *Q. ilex* populations show a wide geographic distribution in the Mediterranean basin. In other words, *Q. ilex* populations analyzed in this study consist of samples from three continents. MP dendrogram based on matK gene-partial trnK gene intron sequences separated the *Q. ilex* populations into three main groups (Figure 1).

The populations resolved in Group I showed two separate clusters: a and b. Cluster a consists of the populations from Croatia, Italy, and France, while cluster b consists of the samples from Albania, Croatia, island populations (Sardinia and Zafferana Etna) of Italy with Apulia, France, and Malta (another island population) that has the very close locality to Zafferana Etna. Group II consists of the samples collected in Morocco, three populations of Algeria, five populations of Spain, and three population of France and Greece.

Table 3. The information of the *Q. ilex* populations examined is based on matK gene-partial trnK gene intron and ITS1-5.8S rRNA gene-ITS2 sequences.

Çizelge 3. matK geni-kısmi trnK gen intronu and ITS1-5.8S rRNA geni-ITS2 sekansları temelinde incelenen *Q. ilex* populasyonlarının bilgileri.

DNA regions	Pop. (Number)	Alignment length (bp)	Variable site	Parsim-info site	Transitional substitutions (%)	Transversional substitutions (%)	Transition/Transversion Purine (k_1)	Pyrimid. (k_2)	Overall (R)	Nucleotide frek. (%) A+T/U G+C
matK gene-trnK intron	37	*695*	9	8	60.01	39.99	0.17	5.84	1.27	68.09/31.91
ITS1-5.8S-ITS2	20	604	58	32	83.81	16.19	8.34	12.27	4.81	36.78/63.22

Table 4. *Q. ilex* populations and variable sites belonging to a) matK gene-partial trnK gene intron sequences b) ITS1-5.8S rRNA gene-ITS2 sequences (The numbers show variable nucleotides).

Çizelge 4. Q. ilex populasyonları ve varyasyonlu nükleotid bölgeleri a) matK geni-kısmi trnK gen intron sekansları b) ITS1-5.8S rRNA geni-ITS2 sekansları (Numaralar varyasyonlu nükleotidleri gösterir).

a)

	1 3 4 4 4 4 4 5 5
	4 1 0 2 3 3 5 6 8
	0 3 2 6 1 9 5 0 9
Quercus ilex (Italy-1)	- T A G T C C G A
Quercus ilex (Türkiye-1)	C T T G A T T G A
Quercus ilex (Italy-2)	- T A G T C C G A
Quercus ilex (Türkiye-2)	C T T G A T T G A
Quercus ilex (Greece-1)	C T T G A T T A A
Quercus ilex (Spain-1)	T C T T C C C G A
Quercus ilex (Algeria)	T C T T C C C G A
Quercus ilex (Albania)	- T A G C C C G A
Quercus ilex (Greece-2)	T T T T C C C G A
Quercus ilex (Croatia-1)	- T A G T C C G A
Quercus ilex (Italy-3)	- T A G C C C G A
Quercus ilex (Spain-2)	T C T T C C C G A
Quercus ilex (Malta)	- T A G C C C G A
Quercus ilex (Türkiye-3)	C T T G A T T G A
Quercus ilex (France-1)	- T A G C C C G A
Quercus ilex (France-2)	- C T G C C C G A
Quercus ilex (Algeria/Souk)	- C T T C C C G A
Quercus ilex (Algeria/Mascara)	- C T T C C C G A
Quercus ilex (Spain/Coll de Corniols)	- C T T C C C G A
Quercus ilex (Spain/Mallorca)	- C T T C C C G A
Quercus ilex (Italy/Zafferana Etna)	- T A G C C C G A
Quercus ilex (Greece/Skyathos)	- T T G A T T G A
Quercus ilex (Greece/Ikaria)	- T T G A T T G A
Quercus ilex (Greece/Drymaia)	- T T G A T T G A
Quercus ilex (Greece/Crete)	- T T G A T T A A
Quercus ilex (France/Lacanau)	- C T T C C C G A
Quercus ilex (France/Rennes-le-Chateaux)	- C T T C C C G A
Quercus ilex (France/Nice)	- T A G C C C G A
Quercus ilex (France/Olmeto)	- T A G T C C G A
Quercus ilex (Croatia/Split)	- T A G T C C G A
Quercus ilex (Croatia-2)	- T A G C C C G A
Quercus ilex (Spain-3)	T C T T C C C G A
Quercus ilex (Italy-Apulia)	- T A G C C C G A
Quercus ilex (Italy-Sardinia)	- T A G C C C G A
Quercus ilex (Italy-Latium)	- T A G T C C G A
Quercus ilex (Morocco/Tangers)	T C T T C C C G A
Quercus ilex (Israel/Mt. Tabor)	C T T G C C C G C

b)

																		1	1	1	1	1	1	1	1	1	1	1	1	1
	1	2	2	2	4	5	6	6	7	7	7	8	8	8	9	9	0	1	1	1	3	3	3	3	4	5	6	6	6
	8	7	8	9	9	2	3	8	3	5	8	1	4	8	0	9	1	3	4	9	0	2	4	7	0	1	2	3	8
Quercus ilex (Italy/Basilicata)	A	A	C	A	G	A	T	A	C	C	C	C	C	A	T	C	C	T	T	C	G	T	G	C	G	C	A	C	G
Quercus ilex (Türkiye/Northern Türkiye)	G	G	.	G	.	G	.	G	.	T	.	.	.	G	C	.	T	C	G	.	C	C	.	T	.	T	.	G	.
Quercus ilex (France/Provence)	G	G	.	G	.	G	.	G	.	.	.	T	A	G	C	.	.	C	G	.	C	C	.	.	.	.	.	G	.
Quercus ilex (Morocco-1)	G	G	T	G	.	G	C	G	.	.	.	T	A	G	C	.	.	C	G	.	C	C	A	.	.	.	.	G	.
Quercus ilex (Morocco-2)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	T	.	C	G	.	C	C	.	.	.	.	.	G	T
Quercus ilex (Spain/Arboretum El Bosque)	G	G	.	G	A	G	.	G	T	.	G	T	A	G	C	.	.	C	G	.	T	C	.	.	.	T	.	G	.
Quercus ilex (Spain/Andalucia)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	T	.	C	G	.	C	C	A	.	.	.	.	G	T
Quercus ilex (Greece/Mainland Greece)	G	G	.	G	.	G	.	G	T	.	G	T	A	G	C	.	.	C	G	T	T	C	.	.	.	T	G	G	.
Quercus ilex (Spain/Huesca)	G	G	.	G	A	G	.	G	T	.	G	T	A	G	C	.	.	C	G	.	T	C	.	.	.	T	.	G	.
Quercus ilex (Spain/Sierra de Tolono)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	T	.	C	G	.	C	C	.	.	.	.	.	G	T
Quercus ilex (Greece/Corfu)	G	G	.	G	.	G	.	G	T	.	G	T	A	G	C	.	T	C	G	.	C	C	.	T	.	T	.	G	.
Quercus ilex (Italy/Lecce)	G	G	.	G	.	G	.	G	T	.	G	T	A	G	C	.	.	C	G	.	T	C	.	.	A	T	.	G	.
Quercus ilex (Spain/Binifaldo)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	.	.	C	G	.	C	C	.	.	.	.	.	G	.
Quercus ilex (Spain/Algarrobet)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	.	.	C	G	.	C	C	.	.	.	.	.	G	.
Quercus ilex (Spain/Constantina)	G	G	.	G	A	G	.	G	T	.	.	T	A	G	C	.	.	C	G	.	T	C	.	.	.	T	.	G	.
Quercus ilex (France/Brignoles)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	.	.	C	G	.	C	C	.	.	.	.	.	G	.
Quercus ilex (Spain/Pinet)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	T	.	C	G	.	C	C	.	.	.	.	.	G	T
Quercus ilex (France/Corse Island)	G	G	.	G	.	G	.	G	T	.	.	T	A	G	C	.	.	C	G	.	C	C	.	.	.	T	.	G	.
Quercus ilex (Italy/Abruzzo)	G	G	.	G	.	G	.	G	T	.	G	T	A	G	C	.	.	C	G	.	T	C	.	.	.	T	.	G	.
Quercus ilex (Italy/Latium)	G	G	.	G	.	G	.	G	T	.	G	T	A	G	C	.	.	C	G	.	T	C	.	.	.	T	.	G	.

	1	1	1	1	1	2	2	3	3	3	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5
	7	7	8	9	9	7	8	2	4	9	1	2	3	4	7	8	8	9	1	3	3	4	5	5	6	7	7	8	9
	1	9	0	5	6	6	3	0	8	5	0	0	8	6	9	6	9	9	2	0	1	1	1	3	4	4	5	9	6
Quercus ilex (Italy/Basilicata)	A	C	G	C	T	A	G	G	G	C	T	T	G	G	C	T	C	A	A	G	T	C	T	C	G	C	T	T	G
Quercus ilex (Türkiye/Northern Türkiye)	G	.	.	.	C	G	.	A	.	.	C	C	.	.	.	C	.	G	.	.	C	.	A	.	.	.	C	C	.
Quercus ilex (France/Provence)	G	.	.	.	C	G	.	.	.	.	.	C	.	.	.	C	.	G	G	.	C	.	.	.	.	.	C	C	.
Quercus ilex (Morocco-1)	G	T	.	.	C	G	.	.	.	.	.	C	.	.	.	C	.	G	G	.	C	.	.	.	.	.	C	C	.
Quercus ilex (Morocco-2)	G	.	.	.	C	G	.	.	.	.	.	C	A	.	.	C	.	G	.	.	C	A	.	T	.	.	C	C	.
Quercus ilex (Spain/Arboretum El Bosque)	.	.	A	.	.	.	.	.	.	T	.	.	.	A	T	C	.	G	.	.	C	.	.	.	.	.	.	.	.
Quercus ilex (Spain/Andalucia)	G	.	.	.	C	G	A	.	.	.	.	C	A	.	.	C	.	G	.	.	C	A	.	T	.	.	C	C	.
Quercus ilex (Greece/Mainland Greece)	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	G	.	.	C	.	.	.	.	.	.	.	.
Quercus ilex (Spain/Huesca)	.	.	A	.	.	.	.	.	.	T	.	.	.	A	T	C	.	G	.	.	C	.	.	.	.	.	.	.	.
Quercus ilex (Spain/Sierra de Tolono)	G	.	.	.	C	G	.	.	.	.	.	C	.	.	.	C	.	G	.	.	C	.	.	T	.	.	C	C	.
Quercus ilex (Greece/Corfu)	.	.	.	T	C	G	.	.	.	.	.	C	.	.	.	C	.	G	.	.	C	.	.	.	.	.	C	C	C
Quercus ilex (Italy/Lecce)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.
Quercus ilex (Spain/Binifaldo)	G	.	.	.	C	G	.	.	.	.	.	C	.	.	.	C	.	G	.	A	C	.	C	.	.	.	C	C	.
Quercus ilex (Spain/Algarrobet)	G	.	.	T	C	G	.	.	.	.	.	C	.	.	.	C	.	G	.	.	C	.	.	.	.	G	C	C	C
Quercus ilex (Spain/Constantina)	.	.	A	.	.	G	.	.	.	T	.	.	.	A	T	C	.	G	.	.	C	.	.	.	.	.	.	.	.
Quercus ilex (France/Brignoles)	G	.	.	.	C	G	.	.	.	.	.	C	.	.	.	C	.	G	.	.	C	.	C	.	.	.	C	C	.
Quercus ilex (Spain/Pinet)	G	.	.	.	C	G	.	.	.	.	.	C	A	.	.	C	.	G	.	.	C	A	.	T	.	.	C	C	.
Quercus ilex (France/Corse Island)	G	.	.	.	C	G	.	.	.	.	.	C	.	.	.	C	.	G	.	.	C	A	.	T	A	.	C	C	.
Quercus ilex (Italy/Abruzzo)	.	.	.	.	C	G	.	.	A	.	.	C	.	.	.	C	G	.	.	.	C	.	.	.	.	.	C	C	.
Quercus ilex (Italy/Latium)	.	.	.	.	C	G	.	.	A	.	.	C	.	.	.	C	.	G	G	.	C	.	.	.	.	G	C	C	.

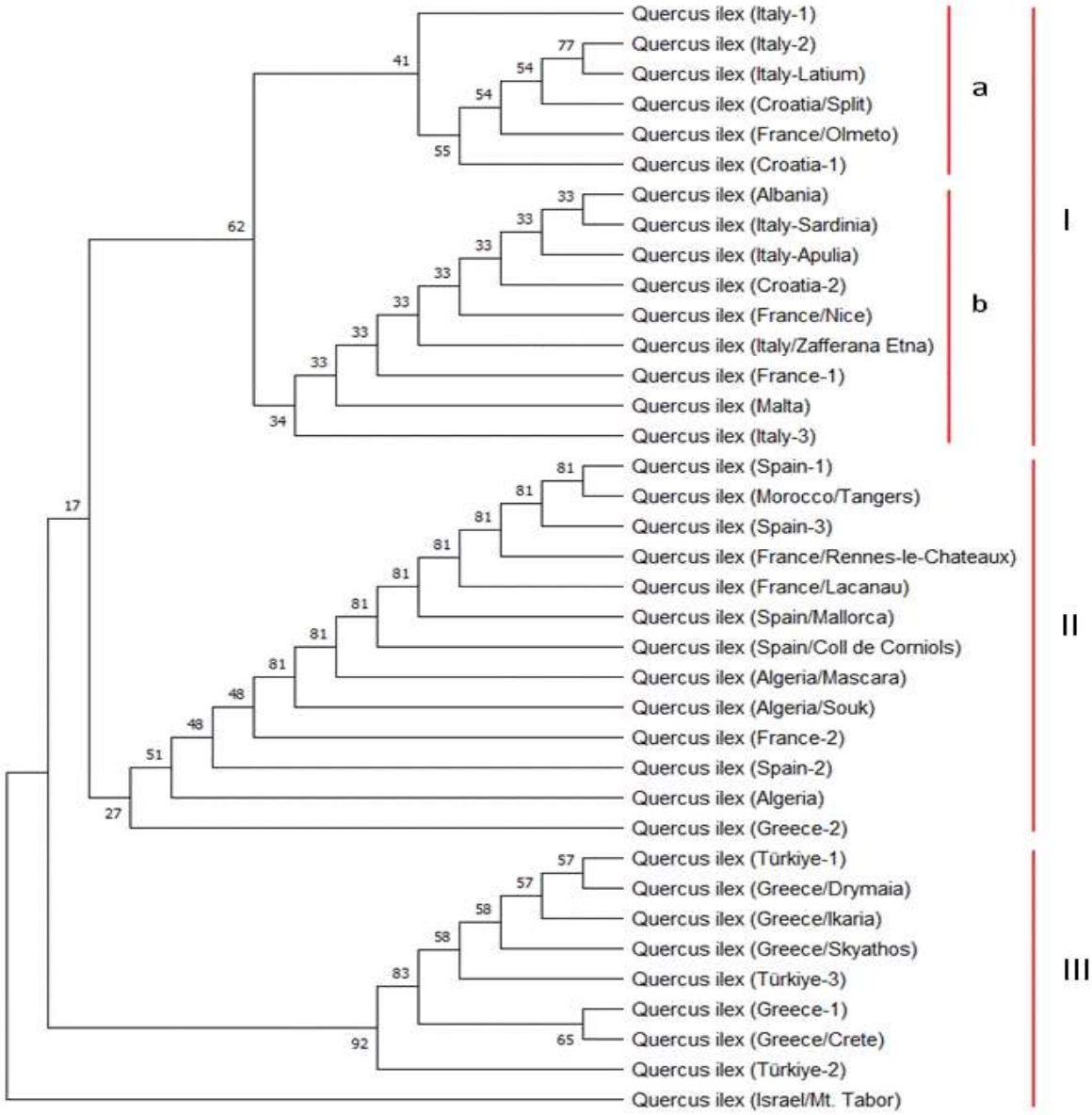

Figure1. Maximum Parsimony tree provided from matK gene-partial trnK gene intron sequences of *Q. ilex* populations.

Şekil 1. Q. ilex populasyonlarının matK geni-kısmi trnK gene intron *sekanslarından elde edilen MP ağacı.*

In summary, populations from dry distribution areas of the Mediterranean climate such as North Africa, alongside samples from Spain, were clustered together, forming a separate group in dendrogram. Additionally, the populations from Albania to France generate Group I, distinct from Spanish and African populations (Morocco and Algeria). However, French populations were observed in both Group I and II, and they exhibited the highest variations compared to the other populations.

Hybridization and introgression are commonly observed mechanisms in the genus *Quercus,* especially in overlapping zones due to weak reproductive barriers (Kremer et al., 2002; Borazan & Babaç; 2003; Petit et al., 2003). Furthermore, these mechanisms give rise to morphological variations and later make it hard to identify the taxa because of insufficient diagnostic morphological characters. Similar behaviors for *Q. ilex* have been observed and reported by many researchers (Jimenez et al., 2004; Schnitzler et al., 2004; de Casas et al. 2007; Lopez de Heredia et al., 2007; Ortego & Bonal, 2010; Lopez de Heredia et al., 2018). In this concept, the variations that were related to hybridization and introgression, in addition to the variations caused by different climatic and ecological factors in the populations showing wide geographical distribution are observed and different morphological types (rotundifolia and ilex) are defined within the *Q. ilex*. The distribution areas of rotundifolia morphotype are stated by dry climatic Mediterranean areas of North Africa and Spain (Tutın et al., 1964; Vázquez Pardo et al., 2002; Peguero-Pina et al., 2014), while regions between Greece and French Riviera are evaluated as distribution areas

for ilex morphotype (Lumaret et al., 2002; Peguero-Pina et al., 2014). Some regions, such as southern France and, eastern and northern Spain, contain samples with intermediate morphotypes between rotundifolia and ilex (Michaud et al., 1995; Lumaret et al., 2002). However, the distribution areas and the taxonomic status of morphological types are not still completely resolved, due to the evaluation of the variations within the *Q. ilex* in terms of only their distribution areas and morphological characters. This study provides important data to evaluate the variations among *Q. ilex* populations from different geographic regions based on nucleotide sequences that consist of both nuclear and chloroplast DNA. Moreover, thus it is aimed to provide more informative results about morphological types. MP tree based on matK gene-partial trnK gene intron sequences separated the *Q. ilex* populations into Group I (Albania, Croatia, France, and Malta) and Group II (North Africa and Spain). This result supports the separation of *Q. ilex* populations based on morphological types. Furthermore, the distribution areas of the rotundifolia and ilex morphotypes, as stated by many researchers, align with the study results. The samples with intermediate morphotypes that were defined by Michaud et al. (1995) and Lumaret et al. (2002) are characterized by the distribution areas such as south France and coastal areas of eastern and northern Spain. Similarly, *Q. ilex* populations in France were observed in both Group I and II (Figure 1). A total of eight populations from Türkiye and Greece formed a distinct group with the outmost species in the phylogenetic tree and they were clustered together in Group III exhibiting higher variations in comparison to the other populations. The single population from Israel merged from outermost to the clade consisting of Türkiye and Greece populations, showing the highest variation in the phylogenetic tree.

MP dendrogram based on ITS1-5.8S rRNA gene-ITS2 sequences separated the *Q. ilex* populations into two main groups (Figure 2).

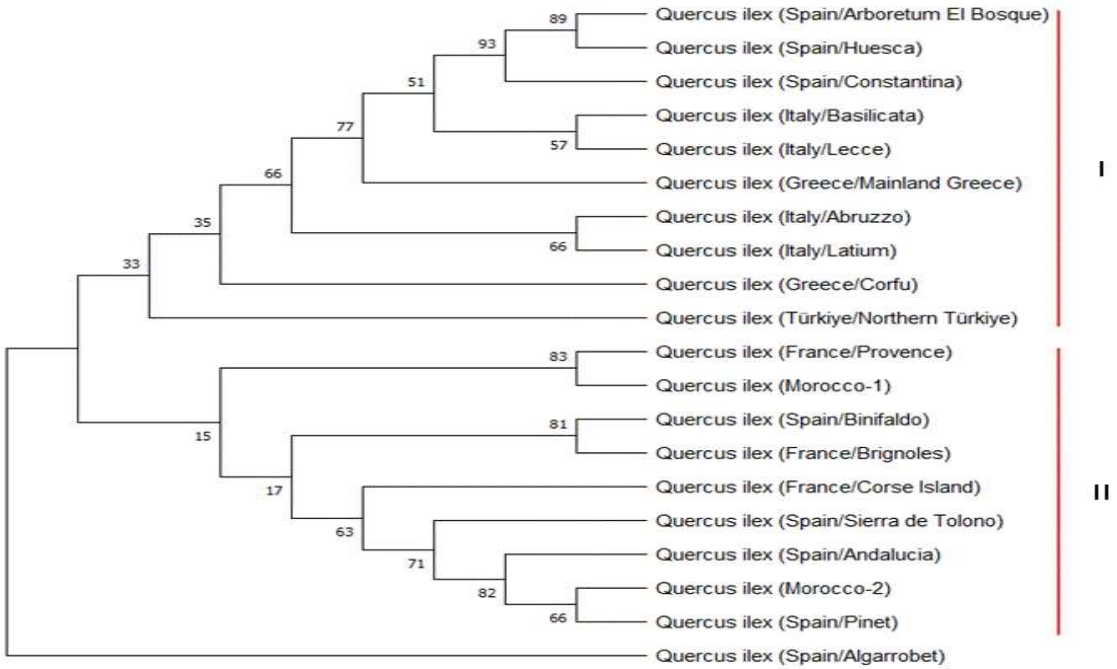

Figure2. Maximum Parsimony tree provided from ITS1-5.8S rRNA gene-ITS2 sequences of *Q. ilex* populations.
Şekil 2. Q. ilex populasyonlarının ITS1-5.8S rRNA geni-ITS2 *sekanslarından elde edilen MP ağacı.*

The samples resolved in Group I consist of ten populations from Spain, Italy, Greece, and Northern Türkiye. The Spanish populations in Group I are from the interior regions of Spain. Also, it can be stated that the populations were clustered in a phylogenetic tree according to the distribution areas.

Group II consists of the samples collected from two populations in Morocco, three populations in France, and five populations in Spain. Provence and Brignoles are two populations that have close distribution areas on the Mediterranean coast of South France. Corse Island is another population from France that lies southeast of the French mainland on the Mediterranean Sea. These France populations were clustered together with Moroccan populations characterized by other dry distribution areas of the Mediterranean climate (Figure II). Similarly, the populations that have distribution areas on the Mediterranean coast of Spain such as Andalucía, Pinet, and Algarrobet (island population) were clustered in Group II.

The MP tree provided from ITS1-5.8S rRNA gene-ITS2 sequences separated the *Q. ilex* populations characterized

by dry Mediterranean climate into Group II. In this concept, the separation of *Q. ilex* populations aligns with the distribution areas of the rotundifolia morphotypes noted by many researchers (Tutın et al., 1964; Lumaret et al., 2002; Vázquez Pardo et al., 2002; Peguero-Pina et al., 2014. However, further studies with more samples from the wide distribution areas in the Mediterranean region are necessary for support.

Both barcoding regions of the chloroplast and nuclear DNA support the separation of *Q. ilex* populations based on different morphological types. Especially, matK gene-partial trnK gene intron sequences in the aspect of the ability to reveal the variations and phylogenetic relationships between *Q. ilex* populations exhibited more clear and informative results than ITS1-5.8S rRNA gene-ITS2 sequences. Additionally, matK gene-partial trnK gene intron sequences have lower sequence variations among populations examined than the region containing ITS sequences.

Therefore, matK gene-partial trnK gene intron sequences are strongly recommended for further studies to reveal variations within *Q. ilex* more clearly and in detail, including samples from all distribution areas. It should also be noted that there are many problems related to the data in NCBI, such as missing habitats and country information in the database. The taxon such as *Q. ilex* which has a wide distribution area is under the influence of different ecological and climatic conditions causing variations. In other words, habitat information of the samples collected from different geographical regions is very important to evaluate variations within the taxon. Deficiencies in this sense complicate interpretation and lead to mistakes in evaluating results. Furthermore, Türkiye is another important region for *Q. ilex* populations with distribution areas that consist of northwest parts and the limited coastal areas of the Black Sea (Yılmaz et al., 2013; Yılmaz et al., 2017). The molecular diversity of *Q. ilex* and their phylogenetic relationships with evergreen oaks in Türkiye were evaluated by Yılmaz et al. (2013). Nevertheless, there is not still enough information about the genetic diversity of *Q. ilex* based on the population genetics. This makes it necessary to conduct studies including the *Q. ilex* populations collected from all distribution areas to obtain more comprehensive and effective results. Finally, DNA sequences preferred in the evaluation of phylogenetic relationships and the determination of genetic diversity have a highly important role in the success of the study, due to variability in species identification and separation ability of the same barcoding region in different plant groups. In other words, it is very important to determine the barcoding regions giving the most accurate and consistent results for the plant group examined. In this concept, although both barcoding regions that consist of matK gene-partial trnK gene intron and ITS1-5.8S rRNA gene-ITS2 sequences provide significant information regarding variations between *Q. ilex* populations, DNA sequences belonging to matK gene-partial trnK gene intron is particularly recommended for their ability to reveal the diversity between populations more clearly, detailed, and meaningfully.

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Author's Contributions

The authors contributed equally.

Statement of Conflict of Interest

The author has declared no conflict of interest.

Appendix

FM244453, FM244439, FM244427, FM244422, FM244411, FM244365, FM244363, FM244350, FM244344, DQ342360, DQ342359, DQ342358, DQ342356, DQ342355, DQ342354, DQ342353, DQ342351, DQ342350, AY226837, AY226836, LT222296, LT222295, LT222294, LT222292, LT222291, LT222290, LT222289, LT222288, LT222285, LT222283, LT222281, LT222278, LT222272, LT222271, LT222269, LT222268, LM652956, LM652955, LM652954, LM652953, LM652952, LM652951, LM652950, LM652949, LM652948, LM652947, LM652946, LM652945, LM652944, LM652943, HE583659, HE583656, HE583624, HE583623, HE583622, HE583620, HE583616.

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