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Molecular phylogenetic analysis of the taxa belonging to the genus *Carlina* L.(*Asteraceae*) in Turkey

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Abstract

Asteraceae, is a cosmopolitan family in terms of natural chemistry, flowering morphology, and adaptation to habitat. Even if there are numerous taxonomic and new molecular phylogenetic studies, the ancestors of the Asteraceae family are not well defined. Study material under consideration, Carlina L., is a member of the tribe Cardueae which belong to Cichorioideaea subfamily of the Asteraceae. The genus Carlina is represented by C. lanata, C. vulgaris, C. tragacanthifolia, C. biebersteinii, C. intermedia, C. oligocephala, C. involucrata subsp. libanotica, C. corymbosa, C.graeca in The Flora of Turkey. In this study, molecular phylogenetic analysis of the genus Carlina, which has a natural distribution in Turkey, has been made. The DNA isolation was performed using phenol- chloroform- isoamylalcohol. ITS (Internal Transcribed Region) of the nuclear ribosomal DNA (nrDNA) and the trnL-F (Transfer RNA coding) region of the chloroplast DNA sequences were used as molecular markers. Carlina was selected as ingroup taxon and Cardopatium, Atractylis and Carthamus were outgroup taxa. Taxa belonging to the genus Carlina distributed in Turkey were analyzed phylogenetically using Branch-and-Bound algorithm with maximum parsimony criterion. Data set analysis using 952 best parsimony trees was made. One tree topology was the most reliable and clads have strongly Bootstrap support.

Keywords: *Asteraceae, Cardueae, ITS, trnL-F, Phylogenetic Analysis*[†]

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Türkiye'deki *Carlina* L. (*Asteraceae*) cinsine ait taksonların moleküler filogenetik analizi

Özet

Asteraceae, doğal kimyası, çiçeklenme morfolojisi ve habitata uyumu açısından kozmopolit bir familyadır. Çok sayıda taksonomik ve yeni moleküler filogenetik çalışma olsa da, Asteraceae familyasının ataları tam olarak tanımlanmamıştır. İncelenen çalışma materyali, Carlina L., Asteraceae'nin Cichorioideaea alt familyasına ait Cardueae kabilesinin bir üyesidir. Carlina cinsi, Türkiye Florasında C. lanata, C. vulgaris, C. tragacanthifolia, C. biebersteinii, C. intermedia, C. oligocephala, C. involucrata subsp. libanotica, C. corymbosa, C.graeca ile temsil edilir. Bu çalışmada, Türkiye'de doğal yayılış gösteren Carlina cinsinin moleküler filogenetik analizi yapılmıştır. DNA izolasyonu fenol-kloroform-izoamilalkol kullanılarak yapıldı. Moleküler markör olarak nükleer ribozomal DNA'nın (nrDNA) ITS (Internal Transkripsiyonlu Bölge) ve kloroplast DNA sekanslarının trnL-F (Transfer RNA kodlama) bölgesi kullanıldı. İç grup taksonu olarak Carlina, dış grup taksonu olarak Cardopatium, Atractylis ve Carthamus seçilmiştir. Türkiye'de yayılış gösteren Carlina cinsine ait taksonlar, maksimum parsimoni kriteri ile Branch-and-Bound algoritması kullanılarak filogenetik olarak analiz edilmiştir. 952 en iyi parsimoni ağacı kullanılarak veri seti analizi yapılmıştır. Bir ağaç topolojisi en güvenirlikte ve dallar güçlü bir şekilde Bootstrap desteğine sahiptir.

Anahtar Kelimeler: *Asteraceae, Cardueae, ITS, trnL-F, Filogenetik Analiz*

1.Introduction

The Asteraceae family is a cosmopolitan family with natural chemistry, flowering morphology and habitat adaptation. Family contains 1130 genera, nearly 25,000 species and has natural distribution in every region of the world [1].

The family is divided into three sub-families of Barnadesioideae, Cichorioideaea and Asteroideae**.** The study material is Carlina L. species included in the Cardueae tribe of the Cichorioideaea sub-family of the Asteraceae family [2].

The Cardueae tribe is a controversial group and it is divided into 4 subtribes in the traditional classification (Echinopsidinae, Carlininae, Carduinae and Centaurenae) [2]. *Carlina* is included in the Carlininae subtribe. Most problems in the Cardueae tribe are experienced with differentiation of the *Echinopsidinae* and *Carlininae* tribal boundaries [2,3]. The genus *Carlina* is represented by the species in The Flora of Turkey and The East Aegean Island as *C. lanata*, *C. vulgaris*, *C. tragacanthifolia*, *C. biebersteinii*, *C. intermedia*, *C. oligocephala*, *C. involucrata* subsp. *libanotica*, *C. corymbosa*, *C.graeca*, [4] (Figure 1).

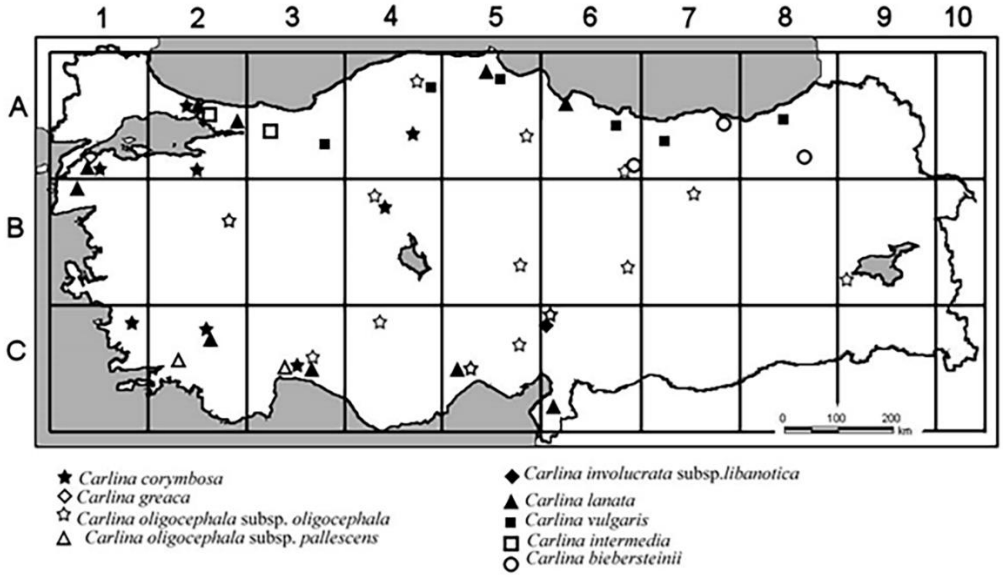

Figure 1**.** Distribution of *Carlina* L. taxon in Turkey [4].

Most *Carlina* species are used for medical purposes. They are used as diuretic, diaphoretic, to increase stomach fluid flow in gastrointestinal disorders and even as mouthwash for colds. In Bulgaria the roots are used for kidney irregularities, skin infections, hemorhoids and nervous disorders. The sap from fresh roots is used as a de-wormer. Roots encourage bile flow and were identified to assist in clearing liver steatosis. Tea used regionally was observed to be good for toothache. Among the public, water prepared from root extracts is known to be effective for washing wounds [5]. It is also recognized as a potent mosquito larvicide [6].

Carlina roots contain 20% inulin, 1-2% volatile oils and flavonoids and these oils have antifungal and antibacterial effects [5].

In spide of studies based on molecular phylogenetics of the *Carlina* species chosen as study material [3,7-9] and some phylogenetic studies performed with morphologic data [10,11], there is no molecular phylogenetic study for species found in Turkey. A study was performed on the pollen morphologies of species found in Turkey [12]. Again, some research used molecular data related to pollen morphology for the Asteraceae family [13,14]. The association between *C. vulgaris* species and *Salix repens* taxon was supported using seedlings of *Salix* taxon grown in humid soil, using water pools in artificial areas on sandy hills and found that they may be suitable areas for *C. vulgaris* mycorrhiza [15]. Research was performed on *C. vulgaris* taxa and population dynamics. Again, apart from the study topic, there is research about the studied taxa (like flora and biomass) in studies [16, 17].

The aim of this study is to research phylogenetic relationship of *Carlina*, a problematic species in systematic terms distributed in the Mediterranean region, with close groups and for taxa it contains. The results will contribute to morphologic differentiation of the *Carlina* species within the Carlininae subtribe, compare molecular phylogeny with traditional classifications within the sub-tribe, determine the boundaries between sub-tribe taxa and analyze contradictions if present, and assess the status of the external group of *Cardopatium*, *Atractylis* and *Carthamus* species in the Cardueae tribe.

In this study, we will try to determine the differentiation limits of the Carlina species and compare the adaptation of the species in Turkey with previous studies.

The study can also be considered as a data source to elucidate the place, distribution area and status of this species in the Turkish flora.

2.Materials and methods

2.1.Plant material, DNA extraction, PCR and sequencing

The study material of *Carlina* L. samples was collected in the vegetation period of the plants in the months from June-September from 2006-2010.

The collected samples were labeled, numbered and dried with herbarium techniques. Leaf and flower portions of samples for use in molecular analyses were dried in silica gel. Similarly, samples for the planned external groups of *Atractylis cancellate, Cardopatium corymbosum, Carthamus lanatus* and *Carthamus dentatus* were monitored during the flowering periods and samples were collected as herbarium material and in silica gel for molecular analyses. Fresh plant samples were prepared as herbarium material with standard herbarium material preparation methods. All samples were given to Balıkesir University Science-Literature Faculty Herbarium after the end of the study.

Firstly, domestic herbariums (Istanbul University Pharmacy Herbarium, Ankara University Herbarium, Gazi University Herbarium, Hacettepe University Herbarium, Ege University Herbarium, Ankara University Pharmacy Herbarium) were visited and available samples were investigated. Additionally, herbariums in Vienna and Geneva were visited. Samples collected in Turkey and neighboring countries in these herbariums were investigated.

Analyses were performed with molecular methods on samples obtained as herbarium material. With this aim, primarily buds, and if not present healthy and green leaves, were used for genomic DNA isolation was performed with the phenol-chloroform-isoamylalcohol method, as the ready-made kits prepared with this match did not give good results [18]. Samples dried in sealed bags containing silica gel were used for isolation. Molecular studies identified the internal transcribed spacer (ITS) and *trn*L-F sequences (Table 1). ITS sequencing used the whole ITS region coding ribosomal RNA belonging to the core genome (ITS1+5.8S rRNA coding DNA+ITS2).

With the aim of supporting these analyses, *trn*L-F primers were studied for chloroplast DNA (cpDNA) sequence variations. These primers are frequently used to determine interspecific relationships of angiosperms [19-21].

Table 1. Primers Used in PCR.

Primer		Nucleotide Sequence (5' - 3')	Tm Value	Primer Designer
Forward	ITS 5A	TCCTCCGCTTATTGATATGC	49.9 ˚C	[22]
Reverse	ITS 4	CCTTATCATTTAGAGGAAGGAG	52.1˚C	[22]
Forward	trnL-e	GGTTCAAGTCCCTCTATCCC	51	[19]
Reverse	trnF-f	ATTTGAACTGGTGACACGAG	51	[19]

For ITS primers, the stages of the PCR program thermocycler program used to proliferate the target regions of the samples were PCR were 5 min 94 ℃ (denaturation), 30 s 94 ℃, 30 s 53 ℃ (This binding temperature varied depending on the tm values of the primers used.), 45 s 72 ℃ (repeated 40 times in 2nd, 3rd and 4th steps), 10 min 72 ℃ and storage at 4 ℃ after the procedure. For *trn*L-F primers, the stages of the PCR program thermocycler program used to proliferate the target regions of the samples were PCR were 5 min 94 ℃ (denaturation), 45 s 94 ℃, 45 s 51 ℃ (this binding temperature was changes linked to the Tm values of the primers used), 2 min 72 ℃ (repeated 35 times in 2nd, 3rd and 4th steps), 10 min 72 ℃ and storage at 4 ℃ after the procedure.

PCR reactions and purification were performed in Balıkesir University Basic Sciences Research Center Laboratories (BÜTAM), with services purchased for remaining sequencing reactions and purification.

With the aim of ensuring the accuracy of these analysis results, the Sequencher program was used to confirm the accuracy of visual DNA sequences.
The phylogenetic trees for the data of DNA sequences obtained in accordance with parameters suitable for the commonly used Phylogenetic Analysis Using Parsimony (PAUP) 4.0b10 phylogenetic analysis software were created [23-25].

3.Results

Phylogenetic analysis was performed, including basic principles, by bringing together molecular systematic analyses of *Carlina* taxa with natural distribution in Turkey. The trees created by the studies were interpreted by supporting the degree of relationship of *Carlina* taxa with two primer sets.

Using matrices related to ITS and *trn*L-F, separate PAUP analyses were performed. For the Branch- and –Bound research set, optimal criterion was parsimony (MP), addition sequence was furthest, multrees choice was selected, initial 'MaxTrees' setting was 100. If the maximum branch length was zero, branch collapse, criteria polytomies, and topological constraints were not enforced and the tree was defined as rootless.

Bootstrap analyses presented the reliability of branches, supporting statistical analysis of phylogenetic analysis with 1000 repeats.

Before PAUP analyses, the character-based method of parsimony criteria was used for the heuristic search analysis and consensus trees were created. Heuristic analyses were limited to maximum 10000 trees. To assess the branches, the tree-bisection-reconnection (TBR) choice was chosen in the program. All character types were determined to be unordered and equal weight. Strict consensus and bootstrap analyses were performed. The distance-based methods of unweighted pair group method using arithmetic average (UPGMA) and the neighbor joining (NJ) method were used.

Sequencing was performed for 15 taxa with ITS and *trn*L-F primers. PAUP analyses created trees according to the heuristic search criteria performed with parsimony.

Table 2. Situation summary of basic and aggregated datasets in Parsimony Analysis.

Datasets	**ITS+ *trn*L-F**
Total characters	1337
Informative	351
Constant	728
Variable	258
Best number of trees	952
Most reliable number of trees	1
Rearragement	1709

Combined analysis using the 'branch-and-bound' algorithm from the MP criteria in PAUP analysis was based on the 952 best parsimony trees. Of these, 1 provided the most reliable tree topology (Table 2).

Combined parsimony analysis with ITS and *trn*L-F used a total of 1337 criteria. The strict consensus tree determined with heuristic search may be better interpreted for reliable differentiation of branches when discussed with bootstrap analysis. The *C. lanata, C. involucrata* subsp. *libanotica, C. biebersteinii, C. greaca, Carthamus lanatus, Atractylis cancellata, Cardopatium corymbosum,* and *C. oligocephala* subsp. *oligocephala* species comprised a monophyletic group differentiated with 100% full support from other *Carlina* species. Again, these species comprise with best supported branches varying from 70-99% on the bootstrap tree. The species determined as external group of *Atractylis cancellata, Cardopatium corymbosum, Carthamus dentatus* and *Carthamus lanatus,* especially, were differentiated with best support on this tree.

C. corymbosa, Carlina 16509, *C.* subsp. *libanotica, C. vulgaris,* and *C. intermedia* species were differentiated from other species with high reliability of 99%. These species comprising a monophyletic group appeared to create a branch with good reliability varying from 66-96%. These species have straw-color phyllaries, leaves on the stem are ovate-lanceolate or linear-lanceolate with spider-web hairs, and close capitula sizes, displaying morphologic similarities.

In addition to the maximum parsimony method, the distance-based methods of UPGMA and NJ analyses were performed (Figure 3, Figure 4).

As *C. traganthifolia* species is a species only identified on islands, it could not be included in the study. When the natural distribution areas in Turkey for the genus are examined, it appears to spread in all regions. An attempt was made to determine the distribution boundaries of the genus more clearly in the present day for localities of samples observed in the visited herbariums in addition to the localities given in the flora of Turkey. The *C. oligocephala* subsp. *oligocephala* taxa were understood to have broadest distribution.

For *Carlina* species, the rosette and stem leaves are important for differentiation of these species. The lack of these features creates a problem for differentiation. The capitulum, leaf features and hair status of the genus were reviewed. Incomplete identifications were performed for identification of species. Tubulate flower and achene features were added [26]. It was observed that most difficulties were experienced between the *C. corymbosa, C. graeca* and *C. vulgaris, C. biebersteinii* species [20].

As expected, differentiation from *Carlina* species was strongly supported for the external group of *Atractylis cancellata, Carthamus dentatus, Carthamus lanatus* and *Cardopatium corymbosum* species (Figure 2).

C. lanata, an annual pink-flowering species in the flora of Turkey, was differentiated from *Carlina* species supported by molecular data. A sample collected from Kırklareli, defined as *Carlina* 16509 with differences supported by molecular analyses, was separated from *Carlina* species with 100% strong support on the combined tree topology (Figure 2). More comprehensive studies should be performed in Kırklareli and surroundings to determine the place for this sample within the genus.

When *C. vulgaris*, *C. intermedia* and *C. biebersteinii* are observed together, it appears that *C. vulgaris* is the species with shortest rosette leaves and widest stem leaves. *C. intermedia* appears to be the species with shortest capitulum among these species. *C. biebersteinii* has different features from the other species with linear-lanceolate leaves. These close similarities may have affected the overlap in the distribution areas.

When the morphologic features of *C. corymbosa* and *C.graeca* are first examined, they appear very similar. Of these two species, *C. corymbosa* may be differentiated by less divided stem leaves, clear and shorter peak thorn. Additionally, the linear-lanceolate stem leaves of *C. gaeca* make differentiation easy. These close similarities were supported by molecular analyses (Figure 2).

C. involucrata subsp. *libanotica* species displays close morphologic features to *C. corymbosa* and *C. graeca*. It is differentiated by the size of the capitulum and the more pronounced sharp thorns. This differentiation is supported by molecular data confirming the monophyletic relationship of the species (Figure 2).

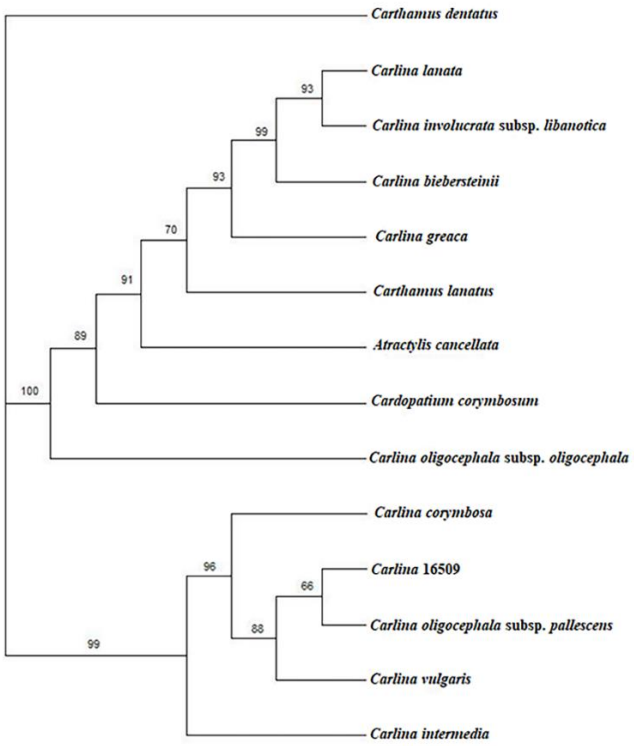

Figure 2. Bootstrap Values on Branch-and-Bound 1 Tree Based on ITS and trnL-F Data Processed Tree Topology.

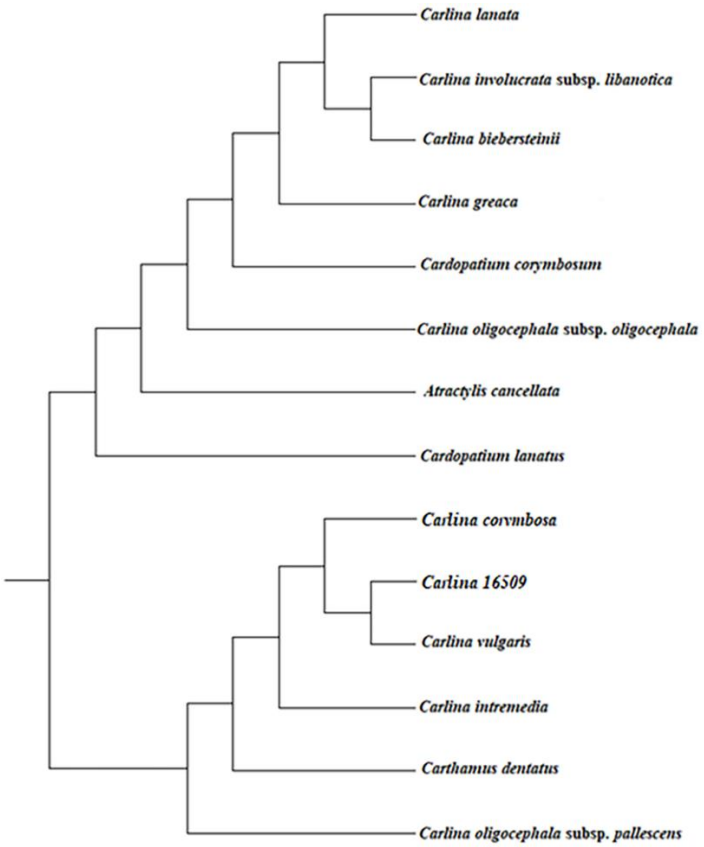

Figure 3. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) Tree Topology Based on ITS and trnL-F Data.

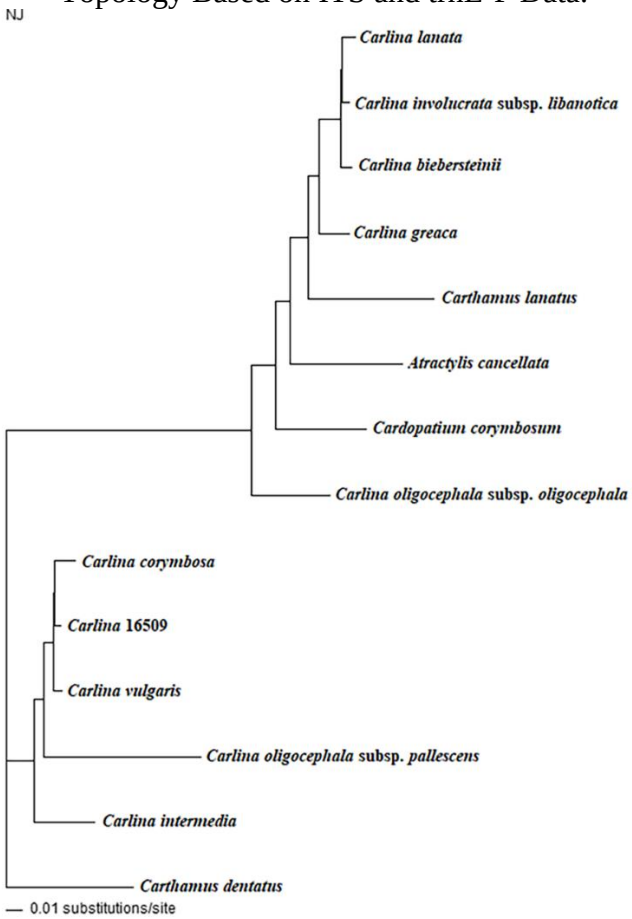

Figure 4. NJ (Neighbor Joining) Tree Topology Based on ITS and trnL-F Data.

4. Discussion

In previous studies, *Carlina* and *Atractylis* were placed as a subtribe of Carlininae [2,7]. *Cardopatium,* placed in the Cardueae tribe, was placed as a subtribe of Carlininae [2]. These taxa are genera located in the Cardueae tribe in the flora of Turkey. Our study supports these results, showing *Carlina* and *Atractylis* are very close species (Figure 2). *Atractylis* appears to be a very close species to *C. vulgaris*, *C. intermedia* and *C. oligocephala* species among *Carlina* species. The *Cardopatium* species was strongly supported by differentiation as an external group close to *Carlina* species, supporting other studies.

C. lanata, which is distinguished by being the only pink-flowered species in the flora of Turkey, is strongly supported in its tree topology. Wahrmund placed this taxon in the Mitina subgenus [27]. It is also included in the *oligocephala* subsp. *oligocephala Carlina*: *oligocephala* subgenus, which is strongly supported and separated.

In the study, the *Carlina* taxa were divided into two clans. The taxa chosen as an outgroup was strongly supported and separated from the *Carlina* taxon. This is in line with the results found by Wahrmund (2010).

It was thought that the sample of *C*. 16509 could be described as a new species or a variation because it differed in morphological observations and molecular analyzes. More comprehensive studies should be performed in Kırklareli and surroundings to determine the place for this sample within the genus. However, the new samples could not be collected due to locality destruction. When the sampling is repeated, the location of the taxa in *Carlina* will be clarified.

Contributions been made to clarifying the place, distribution area and status of these genera in the flora of Turkey in comparison with the results obtained in this study. It is thought that the study will provide additional data to the close studies.

GenBank Submitter: MK238388 (*Carlina vulgaris*), MK238388 (*Carlina oligocephala* subsp *oligocephala*), MK238390 (*Carlina oligocephala* subsp. *pallescens*), MK238391 (*Carlina lanata*), MK238392 (*Carlina involucrata*), MK238393 (*Carlina intermedia*), MK238394 (*Carlina biebersteinii*), MK238395 (*Carlina corymbosa*), MK238396 (*Carlina greaca*)

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