Turkish Salix species: Molecular phylogeny and morphology
Türkiye Salix türleri: Moleküler filogeni ve morfoloji

Pelin Acar¹, Bircan Taşkıran², Funda Ö. Değirmenci²,³, Zeki Kaya²

¹National Botanical Garden of Turkey, Ministry of Agriculture and Forestry, Ankara, Turkey
²Department of Biological Sciences, Middle East Technical University, Ankara, Turkey
³Department of Field Crops, Ahi Evran University Faculty of Agriculture, Kırşehir, Turkey

ABSTRACT
This study provides a new insight into Turkish Salix L. systematics, using a molecular phylogeny and numerical morphometric analysis approach. Despite its economic importance for bioenergy, there is to date no record of any extensive study on this Turkish willow species. Twenty-four Salix species and one hybrid were subjected to molecular and morphometric evaluation, in which one gene region of the external transcribed spacer (ETS) of the 18S-26S nuclear ribosomal DNA and 11 morphological characters were analyzed using a Bayesian Analysis of Beast program and Multiple Correspondence Analysis (MCA) in R. The results indicate that Salix species in Turkey could be accurately classified at the subgenera level, considering the selected gene region and morphological traits (subgenus Salix and Vetrix). Life form, leaf shape (Dim 1) and bud scale (Dim 3) were highly discriminative at the subgenera level. The molecular and morphological data confirmed that the taxonomic position of Salix amplexicaulis needs to be changed as subgenus Salix. Additionally, the members of subgenus Salix, S. acmophylla and S. pentandroides were all clustered distantly from other species of the subgenus.

Keywords: Turkish willows, phylogeny, nrDNA, external transcribed spacer, numerical taxonomy

INTRODUCTION
With over 500 species globally, Salix L. is the largest genus of the Salicaceae (Argus, 1997), occurring mainly in the Northern Hemisphere. There are 65 species in Europe (Kuzovkina and Quigley, 2005) and 27 species in Turkey (Terzioğlu et al., 2014). Four of these 27 Salix species are endemic to Turkey, including S. trabzonica A. Skv., S. purpurea subsp. leucodermis L., S. rizeensis A. Güner et al. J. Zielinski and S. anatolica J. Zielinski and D. Tomaszewski (Güner, 2000; Zielinski and Tomaszewski, 2007). The phytogeographical distributions of some Turkish Salix species correspond to the geographical regions where they are naturally found. For example, Salix aegyptiaca L. (Iran-Turan element) is naturally found in the Southeast Anatolia Region (Avci, 1999). The richest region of Turkey for Salix L. spe-
cies (23 species) is the Black Sea Region, followed by the Eastern Anatolia Region with 15 species. The region with the least number of Salix L. (6 species) is the Southeast Anatolia Region (Anhan and Güvenç, 2011). As members of the Salix genus have small seeds suited for wind dispersion, they can colonize diverse habitats ranging from arid areas to wetlands, from beaches to high mountains (Skvortsov, 1999). In general, systematics data for angiosperms are mainly derived from flower-based characteristics. However, important floral characteristics used in taxonomic studies are absent in Salix species (Azuma et al., 2000), as Salix sp. only has reduced flowers over a very short period in the spring. Therefore, only vegetative traits can be used in Salix systematics, as demonstrated in this study. There are numerous systematic studies on Salix based on morphological traits, which require careful evaluation, as the infrageneric classification of Salix depends on different authors’ treatments. Skvortsov (1999) reviewed Turkish Salix species listed in Davis (1965-1988) and reported the existence of 2 subgenera (Salix and Vetrix) with 13 sections. Despite another recently published paper (Degirmenci et al., 2019), this plant genus is one of the most poorly understood in Turkey.

Economically, Salix species are excellent candidates for bioenergy production (Vermerris, 2008). Some clones of Salix species are used in forest biotechnology for their characteristic quick growth, wide distribution, and resistance to disease and stress (Herrera, 2006). Shrub willows, in particular, have shown to be reliable bioenergy crops, due to their high growth and yield rate in forestry. Willow plantations also mitigate erosion and have a significant impact on afforestation. However, the number of studied willow clones are limited in Turkey (Akgul and Tuctaner, 2011).

The existence of speciation forces within the Salix genus, such as introgressive hybridization, often leads to reticulate taxonomical relations (Azuma et al., 2000; Suda and Argus, 1968). With the increasing problem of uniparental inheritance in phylogeny, rather than cpDNA regions, a number of studies have been conducted on nuclear sequence markers in plant systematics to solve this complex relation. Recently, the nuclear ribosomal DNA ETS gene region has been extensively studied in molecular phylogenetic, due to its high polymorphism rate (Weeks et al., 2004). Although ETS is a short gene region, it was found that ETS sequence data is unique in Salix species (Wu et al., 2015). As traditional methods to identify Salicaceae species using only morphological traits are not sufficient to classify them (due to hybridization, reproductive isolation, and polyploidy), the external transcribed spacer (ETS) of the 18S-26S nuclear ribosomal DNA was sequenced in 26 representative taxa of the Salix L. genus in Turkey. The combination

Figure 1. The locations of sampled Turkish Salix L. species
of molecular sequences and morphometric data based on an appropriate vegetative character set allowed scientists to be familiar to this genus. In this study, the infrageneric problems of 24 Turkish Salix species and one hybrid were studied using molecular and morphometric analysis to bring new insight in Salix taxonomy.

Table 1. The list of Salix species, given codes, and the number of samples representing each species and their location

| The code | Species                  | Subgenus | # Morphological Samples used | District Name/ Province                                      |
|----------|--------------------------|----------|-----------------------------|-------------------------------------------------------------|
| ALBA     | Salix alba               | Salix    | 55                          | Akyazı-Vakıf/Sakarya, Çatak/Konya, Beynam/Ankara, Çoruh/Artvin, Ispir/Erzurum, Bor/Nilüfer, Ürgüp/Neşehir, Ululamak Köprüsü/ Aksaray |
| EXCE     | S. excelsa               | Salix    | 41                          | Çelikli/Samsun Kışlacık Köyü/Kirkkale, Çoruk köyü/Sivas, Yusufeli/Artvin |
| TRIA subsp tri | S. triandra subsp. triandra | Salix     | 29                          | Çerkeş Orman Fidanlığı/Çankırı, Uçköy/Çorum, Tokat, Afyon/Ispir/Erzurum, Tosya-Beğçam/Kastamonu, Ihlara Vadisi /Aksaray |
| TRIA subsp bor | S. triandra subsp. bornmuelleri | Salix     | 2                           | Çeltik-Tersakan/Amasya                                       |
| BABY     | S. babylonica            | Salix    | 22                          | Yalıçay Köyü/Amasya/Ankara, Çoruh-Yaylacak Köyü/Artvin, Kålecik/Ankara, Ihlara vadisi/Aksaray |
| PENT     | S. pentandroide           | Salix    | 6                           | Topulyurdu/Sivas, Beynam/Ankara, Çarşamba/Samsun, Çoruh-Çeşme/Erzurum, Guleman-Aysipinar/Elazığ, Ladik Amasya |
| ALBxfra**| S. alba x fragilis       | Salix    | 1                           | Beynam/Ankara                                               |
| ACMO     | S. acmophylla            | Salix    | 2                           | Asma Köprüsü Suçeken/Batman, Birecik /Şanlıurfa              |
| FRAG     | S. fragilis              | Salix    | 12                          | Çay/Afyon, Beynam/Orman/Ankara, Akşehir/Konya                |
| CINE     | S. cinerea               | Vetricx  | 11                          | Akyazı Gebeş/Sakarya, Çubuk-Karagöl/ANKARA, Çoruh Başbaşi/Erzurum |
| PSEUDO   | S. pseudomedemii         | Vetricx  | 2                           | Zile/Tokat, Beynam/Ankara                                  |
| AEGY     | S. aegyptiaca            | Vetricx  | 2                           | Kars-Erzurum Yolu /Erzurum, Bahçesaray /Van                 |
| WILH     | S. wilhelmsiana          | Vetricx  | 3                           | Kars-Erzurum Yolu /Erzurum, İkizdere/Rize                   |
| VIMI     | S. viminalis             | Vetricx  | 1                           | Nehir Bays/Erzurum                                         |
| PEDI subsp pe | S. pedicellata subsp. pedicellata | Vetricx | 3                           | Göksu-ErmeneK/Karman, Maraş, İkizdere/Rize                     |
| AMPL     | S. amplexicaulis         | Vetricx  | 3                           | Çubuk-Kızılcıhamam/Ankara, Ilgaz/Kastamonu                  |
| ELBU     | S. elbursensis           | Vetricx  | 3                           | Çoruh-Alanbaş/Artvin                                       |
| ARME     | S. armenorossica         | Vetricx  | 2                           | Başbaşi Çoruh/Erzurum                                      |
| ELAE     | S. elaeagnos             | Vetricx  | 3                           | Ilgaz/ Kastamonu                                           |
| CAPR     | S. caprea                | Vetricx  | 3                           | Kızılcıhamam/Ankara, Kastamonu-Çankırı il sinir, Kafkasör Yaylasi/Artvin, Bostan/Kastamonu |
| CAUC     | S. caucasica             | Vetricx  | 3                           | Ayder/Rize, Çoruh-Sıkonaklar/Artvin                        |
| APOD     | S. apoda                 | Vetricx  | 1                           | Ladik/ Amasya                                              |
| PURP subsp leu | S. purpurea subsp. leucodermis | Vetricx | 1                           | Köyceğiz/ Müğla                                          |
| MYRS     | S. myrsinfolia           | Vetricx  | 1                           | Ilgaz/Kastamonu                                            |
| RIZE*    | S. rizeensis             | Vetricx  | 1                           | İkizdere/Rize                                              |
| PSEUDEP* | S. pseudodepressa        | Vetricx  | 1                           | Gümüş Damla Köyü/Bayburt                                   |

*Herbarium species with voucher information
**Hybrid species
MATERIALS AND METHODS

Study Materials
In total, 214 samples of 26 Salix taxa (including one hybrid) from different regions of Turkey were collected and identified (Figure 1). Among these, 45 samples were used to generate molecular data. The codes, sample sizes and locations of each species are provided in Table 1. The topographic and geographic information of samples were provided in more detail in Acar (2017). The duration of field studies for collecting fresh shoots and leaves were limited to the spring and early summer. In the field, shoots with fresh leaves were preserved in packages with silica gel for molecular analyses and pressed for morphological analyses. Herbarium samples of S. pseudodepressa A. Skv. and S. rizeensis from the Hacettepe University Herbarium (HUB) were also analyzed. Unfortunately, the endemic species S. trabzonica and S. anatolica could not be obtained, although field trips were done to record habitats and herbariums were also checked. Populus cathayana was used as an outgroup in our phylogenetic tree. The specimens were identified using the Flora of Turkey and the East Aegean Islands, Vol. 7 (Davis 1965, 1988). Identification issues were resolved by consulting the book by Skvortsov (1999).

Data Collection and Analysis
Nuclear DNA was isolated using the modified Cetyl Trimethyl Ammonium Bromide method from the leaves (Doyle and Doyle 1987). DNA presence and quality were checked and diluted DNA samples (10 ng/µL) were stored at 4°C for a short period. Nuclear ribosomal ETS (Baldwin and Markos, 1998) gene regions were amplified and sequenced using universal primers (at least one sample for each Salix species). PCR amplification was accomplished in 20 µL reactions using the 5X HOT FIRE Pol Blend PCR Mix (with 15Mm MgCl2; Solis Byodyne, Estonia). PCR reactions were performed with: 3 µL PCR Mix, 0.5 µL each primer pair, 4 µL template DNA and 12 µL water in 0.2 mL sterile Eppendorf tubes. The reactions were performed as initial denaturation at 95°C for 5 min followed by of 1 min at 94°C, 1 min at 58°C for annealing, 2 min at 72°C, and followed by a final extension at 72°C for 10 min. Agarose gels in 1% and 1.5% concentrations were used to run PCR samples. The purification and sequencing procedures were performed by the Genoks Molecular Biotechnology Company (Cinnah, Ankara), a European BGI agent. An ABI3730XL 96 capillary automatic sequencer was used for the sequencing of amplified DNA products. The multiple alignment was done using the CLUSTAL W software and Finch TV (Version 1.4.0) developed by the Geopiza Research Team, to view the chromatogram data and to check base positions (Patterson et al., 2004-2006). Molecular parameters were estimated with the MEGA 6.0 software (Tamura et al., 2013). A phylogenetic tree was constructed based on maximum parsimony, maximum likelihood, and Bayesian inference. DnaSP v5 (Librado and Rozas, 2009) was used to get a nexus format file, which was uploaded to BEAUtii software to get an eXtensible Markup Language (XML) file. The phylogenetic tree was created using BEAST version 1.8.4 (Drummond and Rambaut, 2007) under a coalescent tree prior and random starting tree model.
for each partition with four gamma categories, after running it for 10 million generations of the Markov Chain Monte Carlo. Since there is no intraspecific differentiation according to the selected gene region, only one taxon was used to represent one species in the tree. The software Tree Annotator v1.7.5 was used to estimate the maximum-clade-credibility using the Bayesian posterior probability showing the node base statistic. The tree was visualized in the Fig Tree v1.4.3 software (Rambaut, 2016).

Morphological characteristics were identified for inclusion into the morphological dataset. Some of these traits were selectively eliminated based on their non-discriminative features in the Salix genus by consulting the Flora of Turkey (Davis, 1965-1988). As it was difficult to obtain generative parts of the samples, particularly in the herbarium samples, only discriminative vegetative traits were included the final dataset. Morphometric measurements were made in the field, using fresh and herbarium samples, using a Leica MZ16 Fluorescence Stereomicroscope and Leica microscope camera. The data matrix was formed with nine morphological characters belonging to Salix taxa was standardized with binary coding (Table 2). Two more continuous characters: bud angle (Ba) and petiole length (Pl) were generated by measuring characters on photographs processed with a stereomicroscope (Figure 2). Petiole length was measured using three leaves for each individual species, using the average value of the three measurements. These continuous variables were converted to categorical nominal variables using IBM SPSS Statistic (22.0) for Multiple Correspondence Analysis (MCA). MCA is an extension of correspondence analysis which allows the analysis of relationship patterns of several categorical dependent variables (Abdi and Valentin, 2007). Technically, MCA is obtained by using a standard correspondence analysis on an indicator matrix (i.e., a matrix with binary entries). This statistical technique aims to extract important information from the dataset and provides this information as relationships between categorical dependent variables. A morphometric numerical analysis with 11 morphological characters for Turkish Salix genus was carried out with a Multiple Correspondence Analysis (MCA) using the R function “mca” of “FactoMinerR” package (R Core Team, 2014).

RESULTS AND DISCUSSION

Molecular Analysis

The total length of rDNA ETS was 346 bp (Table 3). Polymorphism levels were high in the ETS gene region of the Salix species, at 14/346. All variable sites were informative. The measure of polymorphism of the overall sequences and nucleotide diversity was as high as 0.020. A high level of GC was observed, which is an indicator of high genomic variation in the DNA sequence. Therefore, this suggests that the ETS gene region was quite diverse and characteristically unique for the Turkish Salix species. Twelve variable sites in the ETS sequence were responsible for the divergence of subgenera of Turkish Salix species at 90, 106, 108, 158, 182, 194, 224, 262, 265, 278, 288, and 292th base positions (Table 4). There is no indel (insertion/deletion) for the selected gene region, showing that this is an important function of this region in evolution and conservation of Salix species. The phylogenetic tree constructed with sequence data from the ETS gene region supported two major groups (subgenera Salix and Vetrix) with high posterior probability values (Figure 3). Our results from ribosomal nuclear DNA data supported the classification system of Skvortsov (1999) in which Turkish Salix L. species can be grouped into two subgenera (Salix and Vetrix). Similar clade formations were also reported for Japanese (Azuma et al.,

| nrDNA          | ETS (external transcribed spacer) |
|----------------|-----------------------------------|
| Number of species | 24+1 hybrid*                     |
| Number of total sequences | 45                             |
| Total length (basepairs) | 346                            |
| GC content (%)     | 59.6                              |
| Conserved sites    | 332                               |
| Variable sites     | 14                                |
| Parsimony informative sites | 14                             |
| Number of indels (insertion and deletion) | 0                              |
| Nucleotide diversity | 0.020                           |

*Salix x fragile as hybrid species.
Table 4. Substitution positions in the nrDNA sequence representing the discrimination of two subgenera and the divergence positions of four *Salix* species

| nrDNA ETS (nuclear DNA external transcribed spacer) | Position of base | Subgenus Salix | Subgenus Vetrix | *S. amplexicaulis* | *S. rizeensis* | *S. pentandroides* | *S. acmophylla* |
|----------------------------------------------------|-----------------|----------------|-----------------|-------------------|----------------|-------------------|----------------|
| 90                                                 | C               | T              | C               | C                 | C              | C                 | C              |
| 106                                                | C               | T              | C               | T                 | C              | C                 | T              |
| 118                                                | G               | A              | G               | A                 | G              | G                 | G              |
| 158                                                | T               | C              | T               | T                 | T              | T                 | T              |
| 182                                                | T               | C              | T               | T                 | T              | T                 | T              |
| 194                                                | T               | C              | T               | T                 | T              | T                 | T              |
| 224                                                | C               | G              | C               | G                 | C              | G                 | G              |
| 262                                                | A               | C              | A               | A                 | C              | A                 | A              |
| 265                                                | C               | T              | C               | T                 | C              | C                 | C              |
| 278                                                | A               | G              | A               | A                 | A              | A                 | A              |
| 288                                                | G               | A              | G               | G                 | G              | G                 | G              |
| 292                                                | A               | G              | A               | A                 | A              | A                 | A              |

Figure 3. Best nuclear ribosomal DNA ETS gene tree for Turkish *Salix* sp.

2000), Chinese (Chen et al., 2010) and American *Salix* sp. (Lauren-Moreau et al., 2015). The first group of constructed Beast ETS tree was the subgenus *Vetrix* group, which had four subclades with low posterior values. The first subclade diverging had a high posterior value (0.98) consisting of *S.elbursensis* Boiss.-S. apoda Trautv, *S.pseudodepressa*-S.aegyptica pairs and *S.elaeagnos* Scop. which attach to pairs externally. The second subclade involves the pairs *S.pseudoedememii* E. Wolf - *S.purpurea* subsp. *leucodermis*, *S.armenorossica* A. Skv. - *S.cinerex* L. Additionally, *S.capastra* L. detached to the pair, *S.pedicellata* subsp. *pedicellata* - *S.caucasica* Zucc. The species *S.viminalis* L. was attached from outside to all species of the third and fourth subclades with high posterior...
values of 0.94. In the second group, the subgenus *Salix* included four subclade pairs, *S. triandra* subsp. *triandra* L. – *S. triandra* subsp. *bornmuelleri* (Hausskn.) A. Skv., *S. excelsa* J.F. Gmelin – *S. babylonica* L., *S. amplexicaulis* Bory and Chaub – *S. alba* L., and *S. alba* x *fragilis* – *S. fragilis* L. with the same posterior number of 0.09. Two subspecies of *S. triandra* were placed at the upper position among subg. *Salix* species. Reticulated and complex relationships were found in subg. *Vetrix*, while closely relationships observed in subg. *Salix* members. The extensive polytomy of subg. *Vetrix* was reported in previous studies (Abdollahzadeh et al., 2011; Barkalov and Kozyrenko, 2014). Variable sites with complex relations had a higher detection rate in subg. *Vetrix* than in subg. *Salix* for this gene region. The results of the substitutions at the 90, 106, 118, 158, 182, 224, 262, 265, 278, 288, and 292 th bp positions of *S. amplexicaulis* and at the 90, 106, 158, 182, 194, 262, 278, 288, and 292 th bp positions of *S. rizeensis* were clustered along with the subgenus *Salix* rather than clustering with members of the subgenus *Vetrix*. The appearance of the subg. *Vetrix* members, *S. amplexicaulis* and *S. rizeensis* in the subg. *Salix* group can be explained by the natural hybridization occurring in mixed habitats. Furthermore, the subgenus *Salix* members, *S. acmophylla* Boiss. (106 and 224 th bp) and *S. pentandroides* A. Skv. (262 th bp) placed outside in this group as a result of the substitutions (Table 4).

**Morphometric Analysis**

Our MCA results indicate that different sets of characters are informative for clustering *Salix* taxa in two dimensions (Figure 4). Based on their morphological characters, a two-dimensional configuration of the MCA revealed two major clusters (subg. *Salix* and *Vetrix*) in the analysis (Figure 5). The subg. *Salix* samples were widely distributed and very accessible compared to the subg. *Vetrix*, which includes all endemic *Salix* species in Turkey. The first three dimensions explained 33.3% of the total morphometric variation. The first axis (Dim1) explained 16.9%, the second axis (Dim2) 9.2% and the third axis (Dim3) 7.2% of the total variation. Thus, for the MCA analysis, a two-dimensional MCA solution was considered as the most satisfactory. Considering variables in Dim1, it is clear that life form, bark type, stipule persistence, leaf shape and twig slender had high loading scores. This suggests that these traits are important in the differentiation of species by *Vetrix* (Table 5). Four traits with high loadings in Dim2 were brach habit, decorticated wood, bud angle and petiole length, which are also important features in *Salix* species classification. All discriminant measures were below 0.76, with a maximum value of 0.752 (leaf shape/Ls) for the first dimension (Dim1) and 0.578 (decorticated wood/Dw) for the second dimension (Dim2) (Table 5).

The cluster formations in Figure 4 show that *S. babylonica* (cluster 1), *S. triandra* subsp. *triandra* (cluster 2), *S. excelsa* (cluster 3), *S. fragilis* (cluster 4) and *S. alba* (cluster 5), belonging to subgenus *Salix* were clearly separated by Dim1. Although there were a few individuals which were outside the species’ clusters, the majority of individuals showed consistency in species clustering. In particular,
all representatives of the exotic species *S. babylonica* was grouped into cluster 1 (based on Dim2) to which brunch habit contributed the most. Over 20 samples of *S. triandra* subsp. *triandra* (cluster 2) were distantly positioned from other subg. *Salix* members. *S. triandra* subsp. *bornmuelleri* were located out of cluster 2 but were only represented by a very low sample size. Both Dim1 and Dim2 were important in separating *S. triandra* subsp. *triandra* species from the others. Subgenus *Vetrix* members dominated cluster 6, which consisted of *S. caprea, S. cinerea, S. caucasica, S. myrsinifolia, S. pseudomedemii, S. amplexicaulis, S. wilhelmsiana, S. pedicellata subsp. pedicellata, S. purpurea subsp. leucodermis, S. rizeensis, S. elaeagnos, S. apoda and S. pentandroides*. However, there is a significant overlap with cluster 7, which includes the hybrid species *S. alba x fragilis*. Cluster 7 seems to be located in the mixed zone of subg. *Salix* and subg. *Vetrix* members, and includes both species of the subgenus *Salix* (*S. acmophylla* and *S. pentandroides*) and subgenus *Vetrix* (*S. armenorossica, S. viminalis, S. elbursensis, S. pseudodepressa* and *S. egyptiaca*). Although all samples of *S. acmophylla* were located in the mixed zone, *S. pentandroides* samples were dispersed in both the mixed zone and in the *Vetrix* clusters. Like *S. pentandroides*, *S. amplexicaulis* samples were also nested in both clusters 6 and 7.

In Figure 5, the MCA plot reveals the first two dimensions, showing the differentiation of the two Turkish subgenera (Subg. *Salix* and *Vetrix*) based on morphological data. Each species is represented

Table 5. Summary of characteristics with the highest loadings (*) on the first three dimensions of MCA

| Number | Character                | Dim1 (first axis) | Dim2 (second axis) | Dim3 (third axis) |
|--------|--------------------------|------------------|-------------------|------------------|
| 1      | Lf (Life form)           | 0.660*           | 0.010             | 0.004            |
| 2      | Bs (Bud scale)           | 0.066            | 0.018             | 0.260*           |
| 3      | Bh (Brunch habit)        | 0.046            | 0.505*            | 0.148            |
| 4      | Bt (Bark type)           | 0.576*           | 0.022             | 0.042            |
| 5      | Sp (Stipule persistence) | 0.416*           | 0.004             | 0.024            |
| 6      | Dw (Decorticated wood)   | 0.136            | 0.578*            | 0.008            |
| 7      | Ls (Leaf shape)          | 0.752*           | 0.000             | 0.000            |
| 8      | Lc (Leaf color)          | 0.263            | 0.087             | 0.155            |
| 9      | St (Twig slender)        | 0.651*           | 0.027             | 0.072            |
| 10     | Ba (Bud angle)           | 0.090            | 0.313*            | 0.381*           |
| 11     | Pl (Petiole length)      | 0.062            | 0.463*            | 0.490*           |
by a high sample size in subg. *Salix* members, whilst there were only a limited number of samples for species in subg. *Vetrix* (Table 1). These results indicate that the two subgenera were almost separated within the two MCA dimensions (Figure 5). The binary data such as tree life form, leaf lanceolate shape (for subg. *Salix*) in Dim 1 and pubescence bud scale (for subg. *Vetrix*) in Dim 3 were the dominant characters in subgenera grouping. Most members of subg. *Salix* were clustered at the top-left position, whereas subg. *Vetrix* members are clustered at middle-lower positions by the Dim 1. Additionally, there is a mixture of subg. *Vetrix* with the subg. *Salix* in the intersection zone. The species *S. triandra* subsp. *triandra* was clustered distantly at the top-right of the MCA plot, separated from the members of subg. *Salix*. Such a distinct separation (cluster 2; 2n=2x=38) from the subg. *Salix* members (2n=4x=76) and the top position of the subg. *Salix* members within the molecular tree may be due to different chromosomal rearrangements (Hamza-Babiker et al., 2009). Some limitations should be noted, however, as we only used one nrDNA region and 11 morphological characters to understand and evaluate Turkish willow species. Although further molecular phylogenetic studies will be required to clarify the taxonomic status of willows, our dataset provides the first morphological and phylogenetic analysis using advanced programs on the complex Turkish *Salix* sp.

### The Role of Biogeography for Both Datasets

Biogeographically, the subg. *Salix* dispersed in the continental climate of central and southwestern Turkey, whereas subg. *Vetrix* species adapted to high latitude, altitude and the cool climate of northern Turkey (Figure 1). The clear separation of two subgenera of Turkish *Salix* species was highlighted by the molecular (12 substitutions in nrDNA ETS) and morphological (life form, lanceolate leaf shape and pubescence bud scale) datasets presented in this study. In the subgenera clustering, bud scales with pubescence (one of the morphological characteristics of Turkish *Salix* subg. *Vetrix*) can reduce the grazing and conserve the leaf from damage by solar radiation in habitats with high altitudes (Ehleringer and Bjorkman, 1978). Most of the subg. *Salix* species are characterized by tree-like life forms and lanceolate leaf shapes. The appearance of a distinct lanceolate leaf form in subg. *Salix*, which is widely distributed in Turkey, is inconsistent with taxonomists’ previous morphological classifications (Davis, 1965-1988). These findings are in accordance with Skvortsov’s (1999) statements that subg. *Salix* is a natural and ancient group displaying primitive characteristics, while subg. *Vetrix* includes species characterized by more advanced and recently evolved traits. The reticulate relations and high rate of polymorphism in subg. *Vetrix* also support the occurrence of recently evolved and complex relations (Hardig et al., 2010).

*S. acmophylla* (subg. *Salix*), naturally found in the Eastern part of Turkey, is well allied far from members of subg. *Salix* in both datasets. All *S. acmophylla* samples were gathered from Urfa and Batman (Figure 1). A potential explanation for this distant positioning might be related to the effect of the Anatolian Diagonal, which is an important geographic speciation barrier, causing taxonomic differentiation between subg. *Salix* members (Bilgin, 2011). Another interesting and distant species of subg. *Salix* is *S. pentandroides*: this species was clustered with subg. *Vetrix*, while samples from the Çoruh river and Erzurum were clustered with samples from mixed zone. Those two sampling locations varied in altitude, latitude, and climatic conditions. Since environmental variables have important impacts on *Salix* growth and natural distribution, morphological characters will be selected and expressed differently in diverse habitats (Skvortsov, 1999; Yıldırım and Kaya, 2017). Thus, *S. pentandroides* members were grouped distantly from subg. *Salix* in both datasets. *S. amplexicaulis*, a member of subg. *Vetrix* separated from subg. *Vetrix* groups for molecular and morphological data. The distant appearance of *S. amplexicaulis* may be explained by possible hybridizations with this subg. *Salix* species in mixed habitats. Therefore, we strongly suggest that *S. amplexicaulis* taxonomically need to be merged with subg. *Salix*. As only one herbarium sample represented *S. rizeensis*, more information should be obtained to evaluate the taxonomic position of this endemic species. Extensive hybridization events in *Salix* L. have resulted in intermediate forms of various morphological characters commonly observed in the hybrid species *Salix x fragilis*. The hybrid was located near *S. fragilis* and *Salix ba* in our molecular tree and in the mixed zone in morphological clustering, as expected.

### CONCLUSION

The studied molecular gene region and morphological traits accurately reflected the taxonomic relationships in Turkish *Salix* species. These species are classed into two subgenera in regards to 12 variables sites in the external transcribed spacer of the ribosomal nuclear DNA gene region and three vegetative morphological characters. The first 3 dimensions (Dim1, Dim2, and Dim3) of our morphological data explained 33.3% of the total morphometric variation. The pubescence on bud scale was discriminative for the subgenus *Vetrix* members located in high-altitude habitat, while tree-like life forms and lanceolate leaf shapes were characteristic of subgenus *Salix* members. Results from our molecular data suggest that *S. amplexicaulis*, which is currently in subg. *Vetrix*, should be merged into subg. *Salix*. Subgenus *Salix* members *S. acmophylla* and *S. pentandroides* were classified as a distinct species, in accordance with our molecular and morphological datasets, as a consequence of their biogeographical distribution in Turkey. This study provided novel molecular and morphometric findings to the poorly understood woody genera *Salix* L. and it results showed more useful information than that found in previous literature. In the Turkish *Salix* species, our molecular analysis supported the results from morphological taxonomy. A more comprehensive study covering all Turkish *Salix* species and more genomic regions is necessary to construct an accurate taxonomic classification for *Salix*.

### Ethics Committee Approval: N/A.

### Peer-review: Externally peer-reviewed.

### Author Contributions: Concept – PA, Z.K.; Design – PA, Z.K.; Supervision – PA, Z.K.; Resources – PA, Z.K., F.O.D.; Materials – PA, F.O.D.; Data Collection and/or Processing – PA, B.T., F.O.D.; Analysis and/or Interpretation – PA, B.T.; Literature Search – PA, B.T.; Writing Manuscript – PA; Critical Review – PA, Z.K.; Other – PA, Z.K.

### Acknowledgements: This study was funded by the Scientific and Technical Research Council of Turkey (TÜBİTAK) under project TOVAG...
Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

REFERENCES

- Abdi, H., Valentin, D., 2007. Multiple correspondence analysis. In N.I. Salkind (Ed.): Encyclopedia of Measurement and Statistics, Thousand Oaks (CA)Sage, pp. 651-657.
- Abdollahzadeh, A., Kazempour, O.S., Maassoumi, A., A. 2011. Molecular Phylogeny of the Genus Salix (Salicaceae) with an emphasis to its species in Iran. Iranian Journal of Botany 17(2): 244-253.
- Acar, P., 2017. Molecular Phylogenetics of Turkish Salix L. species (Unpublished Ph.D. Thesis, Middle East Technical University, p. 123, Ankara Turkey).
- Akgül, S., Tunçtaner, K., 2011. Selection of suitable willow clones for biomass production and growing techniques in Turkey, in: Tercer Congreso Internacional de Salicáceas en Argentina, Argentina.
- Argus, G.W., 1997. Infrageneric Classification of Salix (Salicaceae) in the New World. Systematic Botany Monographs 52: 1-121. [CrossRef]
- Anhan, O., Güvenç, A., 2011. Studies on the anatomical structure of stems of willow (Salix L.) species (Salicaceae) growing in Ankara province. Turkish Journal of Botany 35: 535-551.
- Avúc, M., 1999. Türkiye’nin Doğal Söğütleri ve Coğrafi Dağılışları. İstanbul Üniversitesi Edebiyat Fakültesi. Coğrafya Bölümü Coğrafya Dergisi 7: 1-24.
- Azuma, T., Kajita, T., Yokoyama, J., Ohashi, H., 2000. Phylogenetic relationships of Salix (Salicaceae) based on rbcL sequence data. American Journal of Botany 87: 67-75. [CrossRef]
- Baldwin, B.G., Markos, S., 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of ETS and ITS trees of Calycadenia (Compositae). Mol Phylogenet Eval 10(3): 449-63. [CrossRef]
- Barkalov, V.Y., Kozyrenko, M.M., 2014. Phylogenetic relationships of Salix L. subg. Salix species (Salicaceae) according to sequencing data of intergenic spacers of the chloroplast genome and ITS rDNA. Russian Journal of Genetic 50(8): 828-837. [CrossRef]
- Bilgin R., 2011. Back to the Suture: the distribution of intraspecific genetic diversity in and around Anatolia. International Journal of Molecular Sciences 12. 4080-4103. [CrossRef]
- Chen, J., Sun, H., Wen, J., Yang, Y., 2010. Molecular Phylogeny of Salix L. (Salicaceae) Inferred from Three Chloroplast Datasets and Its Systematic Implications. International Association for Plant Taxonomy (IAPT). Taxon 59(1): 29-57. [CrossRef]
- Davis, P.H.R., 1965-1988. In Flora of Turkey and the East Aegean Islands, Vol. 7. 1st ed. - Edinburgh: UK: Edinburgh University Press, pp. 694-716.
- Degermenci, FO, Acar P., Kaya, Z., 2019. Consequences of habitat fragmentation on genetic diversity and structure of Salix alba L. populations in two major river systems of Turkey. Tree Genetics & Genomes 15: 59. [CrossRef]
- Drummond, A.J., Rambaut, A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7(1): 214. [CrossRef]
- Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11-15.
- Ehleringer, J.R., Björkman, O., 1978. A Comparison of Photosynthetic Characteristics of Encelia Species Possessing Glabrous and Pubescent Leaves. Plant Physiology 62: 185-190. [CrossRef]
- Güner, A., 2000. Salix Liri: Güner, A., Ozhatay, N., Ekim, T., Başer, K.H.C. (eds.), Flora of Turkey and the East Aegean Islands. Vol.11.- Edinburgh: Edinburgh University Press, pp. 216-217.
- Hamza-Babiker, N., Berthold, H., Gössl, J., Arnold, C., 2009. Chloroplast DNA identification of eight closely related European Salix species. Austrian Journal of Forest Science 126: 175-193.
- Hardig, T.M., Anttila, C.K., Brunfeld, S.J., 2010. A Phylogenetic Analysis of Salix (Salicaceae) Based on matK and Ribosomal DNA Sequence Data. Journal of Botany 2010: 1-12. [CrossRef]
- Herrera, S., 2006. Bonkers about biofuels. Nature Biotechnology 24(7): 755-660. [CrossRef]
- Kuzovkina, YA., Quigley, M.F., 2005. Willows beyond wetlands: uses of Salix L. species for environmental projects. Water, Air & Soil Pollution 162: 183-204. [CrossRef]
- Laurenou-Moreau, A., Pitre, E.F., Argus, G.W., Labrecque, M., Brouillet, L., 2015. Phylogenetic Relationships of American Willows (Salix L., Salicaceae). PLoS One 10(9): e0138963. [CrossRef]
- Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25(11): 1451-1452. [CrossRef]
- Rambaut, A., 2016. Figtree 1.4.3. Available from: http://tree.bio.ed.ac.uk/software/figtree/. (Accessed: 01 August 2018).
- R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org/.
- Skvortsov, A.K., 1999. Willows of Russia and adjacent countries: Taxonomical and Geographical Revision (transl from: Skvortsov A.K.1968: Willows of the USSR: Taxonomic and Geographic Revision. Nauka, Moscow), ed.: Zinoviev A.G., Argus G.W., Tavaninane, J. & Roininen, H., Joensuu University, Joensuu, p.307.
- Patterson, J., Chamberlain, B., Thayer, D., 2004-2006. Finch TV Version 1.4.0.
- Suda, Y., Argus, G.W., 1968. Chromosome numbers in some North American Salix Britannia 20: 191-197. [CrossRef]
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30(12): 2725-9. [CrossRef]
- Terziçğü, S., Sendar, B., Karakoçê, M., Çoykuçelebi, K., Gültepe, M., 2014. New data on Salix anatolica (Salicaceae) endemic to Turkey. Phytotaxa 167: 111-118. [CrossRef]
- Vermeiren, W., 2008. Genetic Improvement of Bioenergy Crops. Springer, pp. 347-362. [CrossRef]
- Weeks, A., Daly, D.C., Simpson, B.B., 2004. The phylogenetic history and biogeography of fraxinence and myth family (Burseraceae) based on nuclear and chloroplast sequence data. Molecular Phylogenetics and Evolution 35: 85-101. [CrossRef]
- Wu, J., Nyman, T., Wang, D., Argus, G.W., Yang, Y., Chen, J., 2015. Phylogeny of Salix subgenus Salix s.l. (Salicaceae): delimitation biogeography and reticulate evolution. BMC Evolutionary Biology 15: 31. [CrossRef]
- Yildirim, K., Kaya, Z., 2017. Gene regulation network behind drought escape, avoidance and tolerance strategies in black poplar (Populus nigra L.). Plant Physiology and Biochemistry 115: 183-190. [CrossRef]
- Zielinski, J., Tomaszewski, D., 2007. Salix anatolica (Salicaceae), a new species from Turkey. Annales Botanici Fennici 45: 386-388. [CrossRef]