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Molecular phylogenetic analysis resolving the taxonomic discrepancies among *Salix* L. species naturally found in Turkey

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Abstract: Detailed phylogenetic relationships and molecular dating are still quite rare for the complex and diverse genus *Salix* L. Here we focus on the taxonomic status and phylogeny of twenty-six *Salix* taxa naturally found in Turkey using the chloroplast DNA regions (*trn T-F, matK*, and *rbcL*) to unravel the relationship among them. The status of *Salix* species was also checked in the phylogenetic tree constructed with the data from Internal Transcribed Spacer (ITS) region of nuclear gene, including 158 accessions from the GenBank and 126 newly generated sequences of 26 *Salix* taxa (24 species) naturally found in Turkey. The phylogenetic analysis of the sequence data from both the chloroplast (cpDNA) and nuclear (nrDNA) DNA regions enabled a reliable classification of the genus at the subgeneric level (*Salix* and *Vetrix*) with high posterior probability/ bootstrap values as 1/100. The study provides important information on the *Salix* phylogenetic placements and diverging times of *S. pentandroides*, *S.apoda*, *S. armenorossica, S. pseudomedemii*, *S. pedicellata* subsp. *pedicellata*, *S. pseudodepressa*, *S. amplexicaulis,* two subspecies of *S.triandra,* and two endemic species of Turkey (*S. purpurea* subsp. *leucodermis* and *S. rizeensis*) for the first time. Taxonomically, *S. amplexicaulis* and *S. rizeensis* previously classified under the subgenus *Vetrix* were clustered phylogenetically under the subgenus *Salix*. Subgenera *Salix* species appears to be diverged from the subg. *Vetrix* in Eocene (ca. 45.1 Mya) while the estimated divergence times of subg. *Salix* and subg. *Vetrix* dated back to 23.1 and 21.65 Mya, respectively. However, divergence times among species within *Salix* and *Vetrix* subgenera of Turkey seem to be around the Pliocene period. Molecular phylogenetic relationship between *Salix* species native to Turkey and *Salix* species from the world were mainly associated with taxonomic hierarchy, rather than geographic proximity.

Keywords: *Salix* L., cpDNA, nrDNA, molecular phylogeny and dating, Turkey

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Introduction

Salix L. (willows) is the largest genus of Salicaceae occurring mainly in the Northern Hemisphere (Argus, 1997). Willows have a variety of uses ranging from modern phytotherapy (Mahdi et al., 2006; Akyürek & Acar, 2020) to the phytoremediation of anthropogenic factors (Evlard et al., 2014) due to their rapid growth and adaptation to a wide range of environmental stresses (Vermerris, 2008). Furthermore, the rapid growth capability of the trees makes them a promising potential for bioenergy production. Around the world, the number of reported willow species varies from 350 (Skvortsov, 1999) to over 500 species (Fang, 1987; Hardig et al., 2010; Wu et al., 2015). Turkey is a prominent country with respect to the richness of biodiversity including forest trees and shrubs (Kaya & Raynal, 2001). One of the most significant members of the Anatolian riparian forests is Salicaceae species (Skvortsov, 1999; Degirmenci et al., 2019). The Anatolia (Asian part of Turkey), considered as the land bridge between Europe and Asia, has diverse ecosystems created by variable climatic and geographical conditions as well as the geographical barrier (Anatolian Diagonal) run from the north (Gümüşhane-Bayburt) to southwest (the Taurus Mountains) across Turkey (Ekim & Güner, 1986). The Anatolian Diagonal involves mountain ranges divide the Irano-Turanian phytogeographic region of Turkey into the east and west (Mutun, 2016) and causes the differentiation of taxa at the species and subspecies level (Bilgin, 2011).

In comparison with Northern Europe, Turkey was not totally covered by ice sheets during the last glacial period (Erinç, 1978). Thus, Turkey acted as the glacial refuges and reservoirs of biodiversity including Salicaceae species. Today, there are 27 willow species naturally found in Turkey (Acar et al., 2020). The richest region of Turkey for *Salix* species is Northern Anatolia. It is followed by the Eastern and Southeastern Anatolia regions (Arıhan & Güvenç, 2009). Among 27 *Salix* species, four species and/or subspecies are endemic to Turkey, namely *S.trabzonica* A. Skv., *S. purpurea* subsp. *leucodermis* L., *S. rizeensis* A. Güner et J. Zieliński (Terzioğlu et al., 2007), and *S. anatolica* J. Zieliński and D. Tomaszewski (Güner, 2000; Zieliński & Tomaszewski, 2007).

The genus *Salix* is well known as one of the few woody genera with a large number of polyploid taxa. About 40% of the willow species are polyploid having allopolyploid origin (Wagner et al., 2020), ranging from diploid to octoploid species with the basic chromosome number of 19 (Argus, 1997). This high polyploidy level that resulted in *Salix* hybrids was reported to be an important mechanism in evolution (Karrenberg et al., 2002). The nature and origin of hybridization in the genus *Salix* are still unclear due to the lack of studies. Evolutionary forces such as dioecious reproduction, natural formation of hybrids, polyploidy, and introgression seem to generate taxonomical problems in *Salix*, which are still debated worldwide (Kuzovkina et al., 2008; Hörandl et al., 2012; Wagner et al., 2018) and a consensus has not been realized until now. High morphological variability in willows has been resulted in taxonomic confusion (Dorn, 1976). Argus (2000) treated *Chosenia* and *Toisusu* as genera in Salicaceae. However, Skvortsov (1999) categorized them as a subgenus since they have only a few different features. In Skvortsov (1999) system, the genus is divided three subgenera: *Salix*, *Vetrix*, and *Chamaetia*. Ohashi (2001) recognized two additional subgenera (*Chosenia* and *Toisusu*) and further grouped the *Salix* genus into six subgenera. The genus is generally divided into five subgenera: *Salix* subg. *Salix* s. str., subg. *Longifoliae* (Andersson) Argus, subg. *Protitae* Kimura, subg. *Chamaetia* (Dumort). Nasarov in Kom., and subg. *Vetrix* Dumort. (Argus, 2010; Wu et al., 2015; Wagner et al., 2018). Skvortsov (1999) reviewed the *Salix* species native to Turkey and reported the existence of two subgenera (*Salix* and *Vetrix*) and 13 sections. The oldest fossil record of subg. *Salix* and subg. *Vetrix* from North America dated back to Eocene (33.9–56 Million years ago/Mya) and Oligocene (23–33.9 Mya), respectively (Wolfe, 1987). European and Russian *Salix* fossils were recorded in Oligocene (Collinson, 1992).

Traditional methods (morphological identification) of taxonomy are insufficient for identifying the members of the genus *Salix*. In several studies, molecular phylogenetic methods were used to address the phylogenetic issues of the genus, but these studies did not include the *Salix* species native to Turkey (Brunsfeld et al., 1991; Leskinen & Alström-Rapaport, 1999; Azuma et al., 2000; Hardig et al., 2010; Chen et al., 2010; Abdollahzadeh et al., 2011; Wu et al., 2015; Lauren-Moreau et al., 2015; Liu et al., 2016; Wagner et al., 2018; Wagner et al., 2020).

The three non-coding chloroplast DNA (cpDNA) regions *trn T-F* intergenic spacer, the chloroplast coding gene maturase Kinase (*matK*), the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, and the ribosomal internal transcribed spacer (ITS) region of nuclear DNA (nrDNA) are commonly preferred in molecular phylogenetic studies since they provide sufficient data to answer questions in phylogenetic reconstruction and classification (Pirie et al., 2007; Hilu et al., 2003; Savolainen et al., 2000; Alvarez & Wendel, 2003). A recent study on *Salix* species naturally found in Turkey (Acar et al., 2020) stressed the importance of multiple sequences from both nuclear and chloroplast genomes are needed to resolve taxonomic discrepancies present in the

genus and to reveal proper phylogenetic placements of species.

In the current study, we aimed to provide valuable molecular data to fill the gap in *Salix* molecular phylogeny by extensive sampling of *Salix* species native to Turkey and studying both sequences of chloroplast genome (*trn T-F* intergenic spacer, *matK*, *rbcL*) and nuclear genome (ITS). Specifically, the objectives were, (i) to shed a new light on the phylogenetic placement of some taxomically problematic *Salix* species naturally found in Turkey, (ii) to generate and provide DNA sequence data from chloroplast and nuclear genome of those *Salix* taxa to reveal interspecific phylogenetic relationships and molecular divergence time and (iii) to investigate evolutionary lineages of *Salix* species in the world by using ITS sequences available from the GenBank. This novel study unravels the phylogenetic position and molecular dating of some *Salix* taxa sampled across Turkey at first and explored their relation in the world.

Methods

Plant materials

The collection of fresh willow leaves is extremely important for high-quality DNA extraction. Therefore, the sampling period was limited to only from Spring to mid-Summer. The collected fresh leaves were preserved in silica gel until the DNA extraction experiments. For each species, at least two samples were taken from different locations of Turkey (Supplementary Table S1). To identify the species accurately, one of the samples of each species was collected with catkins. One hundred and twenty-six individual samples were taken from field collections and herbarium specimens. Detailed information on sample locations was provided in Fig. 1. The species identifications were made mainly according to the Flora of Turkey (Davis, 1965). Other relevant databases such as Turkish Plant Lists-Vascular Plants

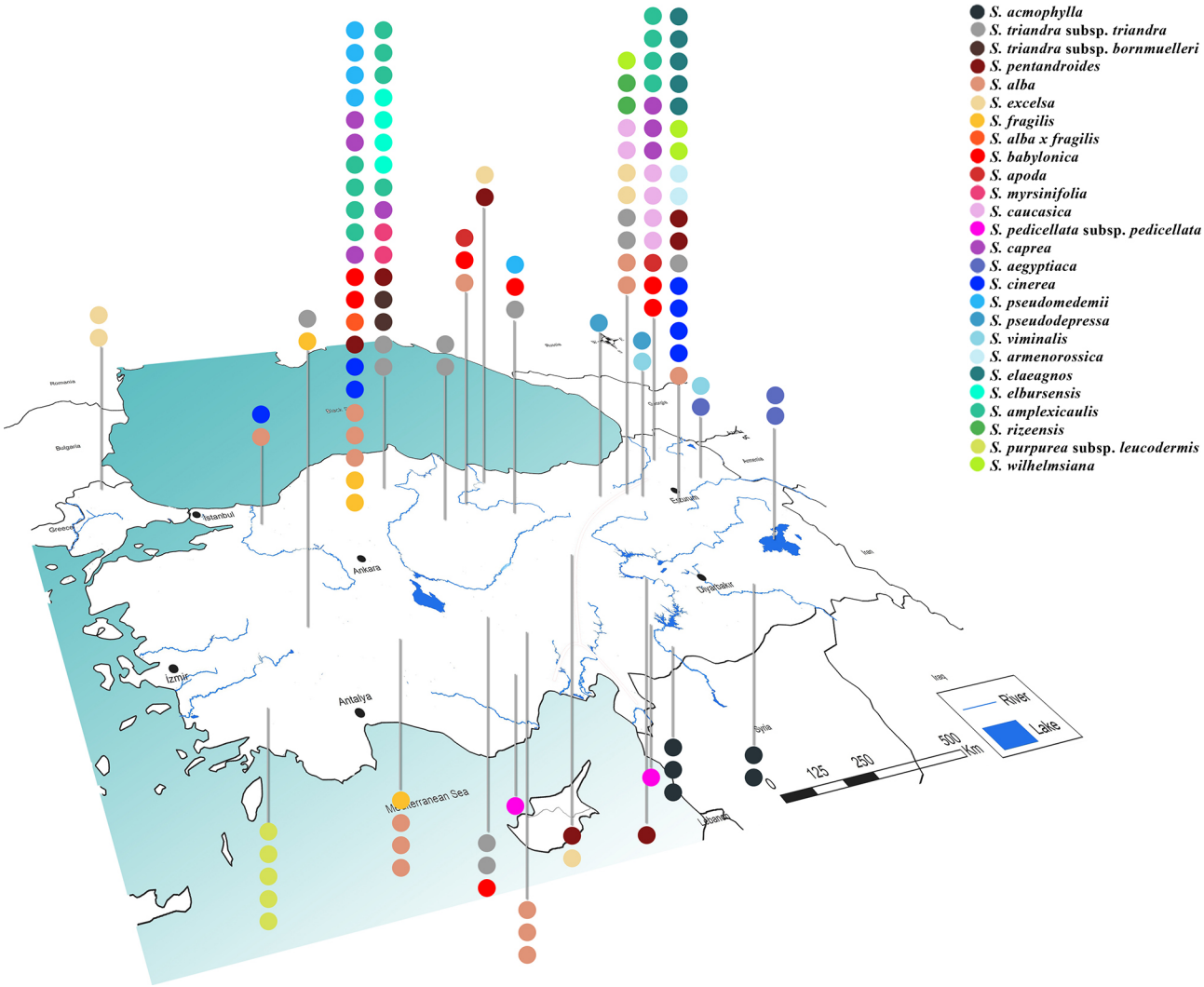

Fig. 1. The locations of each *Salix* species native to Turkey

Table.1 Information on accession code, country, subgenera name and the numbers of taxa used in ITS phylogenetic tree

	Code	Country	Subg. *Salix*	Subg. *Vetrix*	Subg. *Chamaetia*	Subg. *Protitea*	Subg. *Longifoliae*	Subg. *Chosenia*	Total number of taxa used
1	TR	Turkey	7	19					26
2	IR	Iran	9	10					19
3	RU	Russia	1	14	12				27
4	CH	China	13	6	3	2	1		25
5	SW	Sweden	5	3	2				10
6	CA	Canada		3					3
7	AM	America	11	39	16	4	2	1	73
8	SZ	Switzerland		1					1
9	*Populus_Outgroup_CH*	China							14
Total			46	95	33	6	3	1	198

(Türkiye Bitkileri Listesi-Damarlı Bitkiler) (Güner et al., 2012), Turkish Plants Data Service (TUBIVES) checklist (Babaç, 2004) and Willow of Russia and Adjacent Countries (Skvortsov, 1999) were also used for the purpose of species identification and taxonomy. For comparative molecular phylogenetic analysis, the ITS sequences of *Salix* species taxonomically related to Turkish species from the GenBank database (158 accessions) were retrieved to understand the evolutionary divergence and placement of the *Salix* species (Table 1). Majority of accession were from America, Russia, and China covering mainly five subgenera (subg. s.str *Salix*, *Vetrix, Chamaetia, Protitea, Longifoliae* (Table 1)). Details of the downloaded sequences from GenBank were given in Supplementary Table S1.

DNA extraction, amplification and sequencing

Nuclear DNA was isolated from leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). The presence and quality of the DNA were assessed using a spectrophotometer (Biodrop μLite, UK). The DNA isolation procedure was repeated and optimized until a sufficient amount and quality of DNA concentration was obtained to be used in polymerase chain reaction (PCR). The diluted DNAs (10 ng/μl) were stored at 4 °C for a short period, whereas stock DNA samples were stored at −80 °C for a long period. The non-coding *trn T-L* intergenic spacer, tRNA-Leu- *trn L* and *trn L-F* intergenic spacer (*trn T-F*), coding maturase Kinase (*matK*), and coding ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) regions of chloroplast DNA and the ribosomal internal transcribed spacer (ITS) region of nuclear DNA were amplified and sequenced using universal primers (Taberlet et al., 1991; Li et al., 1997; Savolainen et al., 2000; Hsiao et al., 1995) for each of the 126 *Salix* samples coming from 26 *Salix* taxa of Turkey. The PCR amplifications were performed in 20µl reaction mixture which included 3 µl PCR Mix (5X HOT FIREPol Blend PCR Mix solution with 15Mm $MgCl_2$), 0.5µl each primer pair, 4 µl template DNA, and 12 µl water in 0.2 ml sterile Eppendorf tubes. The PCRs were performed using a thermocycler (Eppendorf Mastercycler, Canada) based on the optimized cycling parameters as follows: an initial denaturation at 95 °C for 5 min followed by denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. Agarose gel concentrations of 1% and 1.5% were used to visualize the PCR products. The purification and sequencing procedures were carried out by Genoks Molecular Biotechnology Company (Cinnah, Ankara). The chromatogram data obtained from the sequence analysis were viewed using FinchTV software (Version 1.4.0) developed by the Geopiza Research Team (Patterson et al., 2004–2006). All the DNA sequence base peaks were checked for the accuracy of base calling. The multiple alignments were carried out with the ClustalW software. The sequence assembling and sequence molecular diversity parameters were estimated for all sequence data generated from the chloroplast and nuclear DNA by using the MEGA Software version 6.0 (Tamura et al., 2013).

Phylogenetic analysis

Phylogenetic trees were constructed based on maximum likelihood (ML) and Bayesian inference (BI) analyses. The MrModel software (Nylander, 2004) was used for testing the best suitable substitution model based on the AIC (Akaike information criterion) values. The data were converted to nexus format using DnaSP software (Librado & Rozas, 2009) and to eXtensible Markup Language (XML) format using BEAUti software (Drummond & Rambaut, 2007). The formatted data of cpDNA and nrDNA were analyzed by using the BEAST v 2.5.1 (Bayesian Evolutionary Analysis by Sampling Trees) package program, with the options of GTR+I+G substitution, Yule tree prior and random starting

tree models for each partition with four gamma categories and uniform sampling frequencies. These options were applied by sampling all parameters once every 10,000 generations from 10,000,000 Markov Chain Monte Carlo (MCMC) generations. The poplar DNA sequences of studied cpDNA and nrDNA regions were used as outgroups. The Tracer 1.6 (Rambaut et al., 2014) and TreeAnnotator software (Drummond & Rambaut, 2003) were used to evaluate convergence and to estimate the maximum clade credibility (MCC) tree using Bayesian posterior probability (limit of 1). The Tracer 1.6 was also applied to examine effective sample sizes (ESS) for estimated parameters. For both chloroplast and nuclear sequence data, ESS values were calculated far beyond 200 for both calibrations resulting in reasonable-looking bell-shape posterior probability density curves. Besides, all phylogenetic trees were constructed in MEGA program to estimate bootstrap support values with 1000 replicates in ML option. The bootstrap values (limit of 100) were added to the phylogenetic trees of BEAST analysis (Fig. 2, Fig. 3). The constructed phylogenetic trees with bootstrap values and posterior probabilities of each branches were visualized in the FigTree version 1.4.3 (Rambaut, 2016). Assuming that DNA sequences evolve at a relatively constant rate over time and among different organisms (Futuyma, 2011), a molecular dating analysis of data from cpDNA is useful to understand *Salix* lineage diversification in Turkey. The molecular dating analyses was carried out by the use of BEAST program with the strict molecular clock option. The split between *Salix* and *Populus* as a root node of Salicaceae was assigned to the earliest *Populus* fossil record dated as 48 Mya for calibration (Wu et al., 2015).

Results

Polymorphism

The total sequenced lengths of the ITS, *matK*, *rbcL* and *trn T-F* sequences were 598, 1727, 1485 and 1347 bp, respectively (Table 2). The polymorphism levels were found to be about the same magnitude in both nrDNA (19/598) and cpDNA (123/4550) sequences. Among the studied cpDNA sequences, the *trn T-F* (56/1347) region was the most variable one. The most variable part of *trn* the *T-F* was the *trn L* intron region. The nucleotide diversity as a measure of overall polymorphism was estimated as 0.017 for the non-coding cpDNA *trn T-F* sequence. The variable sites were higher at the sequence of 5' region (*matK*1) compared to the sequences adjacent to the 3' (*matK*2) ends. The most conserved sites were found in the *rbcL* region of cpDNA. The highest transition (79.24), transversion substitution (20.76) and transition-transversion bias (R) (3.51) rates were estimated for the ITS region, which was quite diverse and informative. One insertion (indel) polymorphism was in both ITS and *matK* regions. In general, the ratio of parsimony informative sites to variable sites was higher in nrDNA (14/19) compared to all cpDNA regions (89/123). The nucleotide diversity estimated for both cpDNA and nrDNA sequences was higher in subg. *Vetrix* compared to subg. *Salix*.

Phylogenetic Analysis

The topologies of ITS and chloroplast trees are not totally in coherence, but *Salix* of Turkey was mostly resolved as monophyletic in cpDNA tree (Fig. 2). The genus in the cpDNA tree was separated as two well-supported subgenera (*Salix* and *Vetrix*) along the other smaller clades (1,2,3,4,5). On the

Table. 2 The estimated molecular diversity parameters based on cpDNA and nrDNA sequences of *Salix* species native to Turkey data

	nrDNA	cpDNA			
	ITS	*mat*K	*rbc*L	*trn*T-F	total
Number of species	26 taxa*	26 taxa *	26 taxa *	26 taxa *	26 taxa *
Number of total sequences	126**	126**	126**	126**	126**
Total length (bp)	598	1727	1485	1347	4550
GC content (%)	64.7	32.4	43.3	30.5	35.3
Conserved sites	578	1680	1462	1289	4418
Variable sites	19	45	22	56	123
Parsimony informative sites	14	25	15	49	89
Transitional pairs	79.24	58.79	56.77	50.62	57.23
Transversional pairs	20.76	41.21	43.23	49.38	42.77
Transition/Transversion (tr/tv) (R) ratio	3.51	1.24	1.29	0.86	0.97
Number of indels	1 (insertion)	1 (insertion)	0	0	1(insertion)
Nucleotide diversity	0.013	0.006	0.005	0.017	0.009

**S. alba* × *fragilis* was included in the analysis as a morphologically identified hybrid taxa.*
**Used sequences only from Turkey.

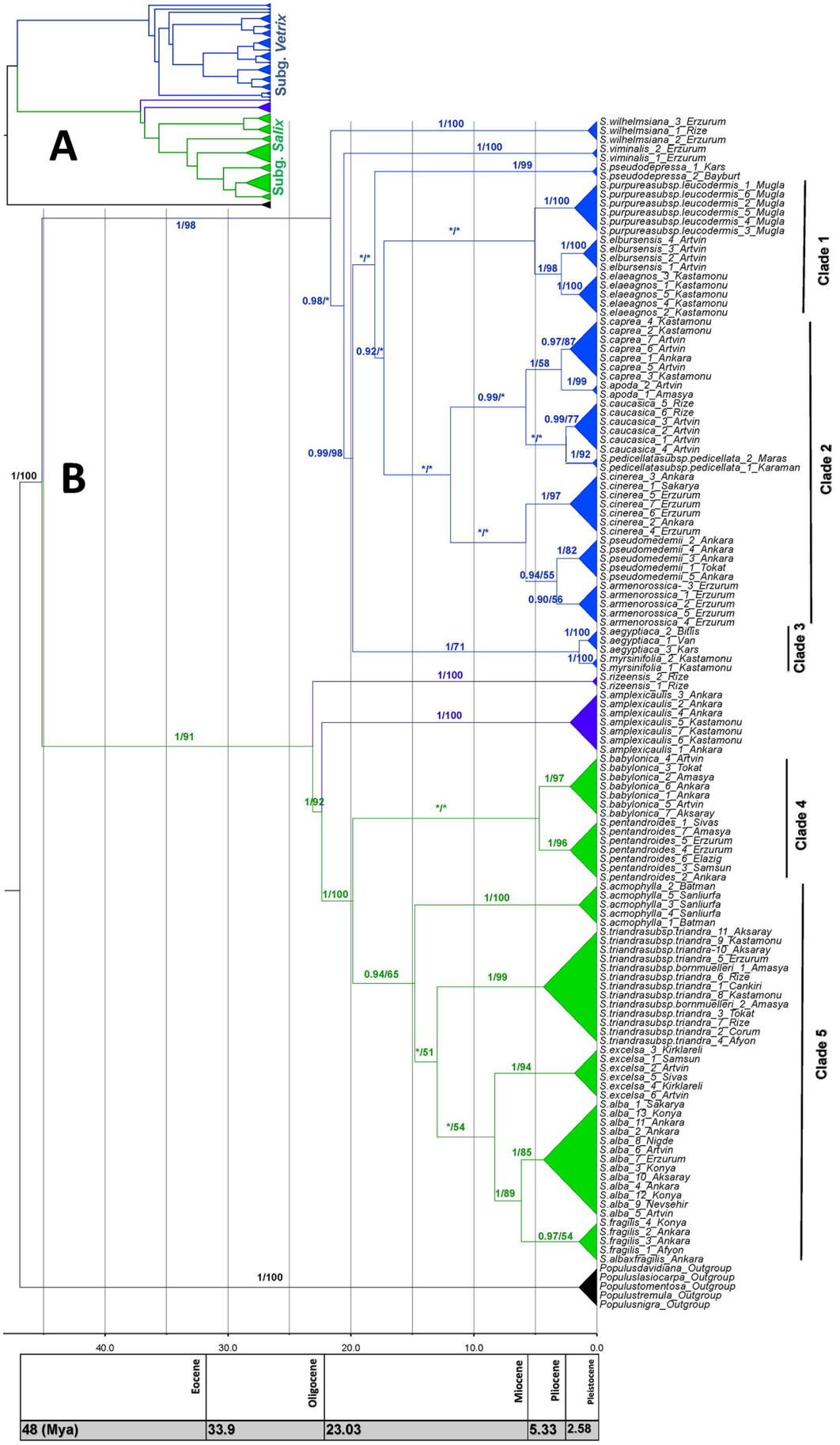

Fig. 2. Beast phylogenetic tree with a Maximum Likelihood and Bayesian molecular dating analysis based on cpDNA sequences (*trn* T-F, *matK*, and *rbcL*) of *Salix* native to Turkey. Different subgenera are depicted using different-colored: Subgenus *Vetrix* is shown in blue and *Salix* is in green (A). Bayesian posterior probability values (up to 1) and ML bootstrap (up to 100) are shown beside branches (pp /bs) where posterior probability ≥0.90 or bootstrap value >50. The low values (pp ≤ 0.90 or bs <50) were given as only asterisks (*). The location of samples used to construct phylogenetic tree are shown behind the species name (B). Values throughout the Cenozoic Era: 48 Mya to present

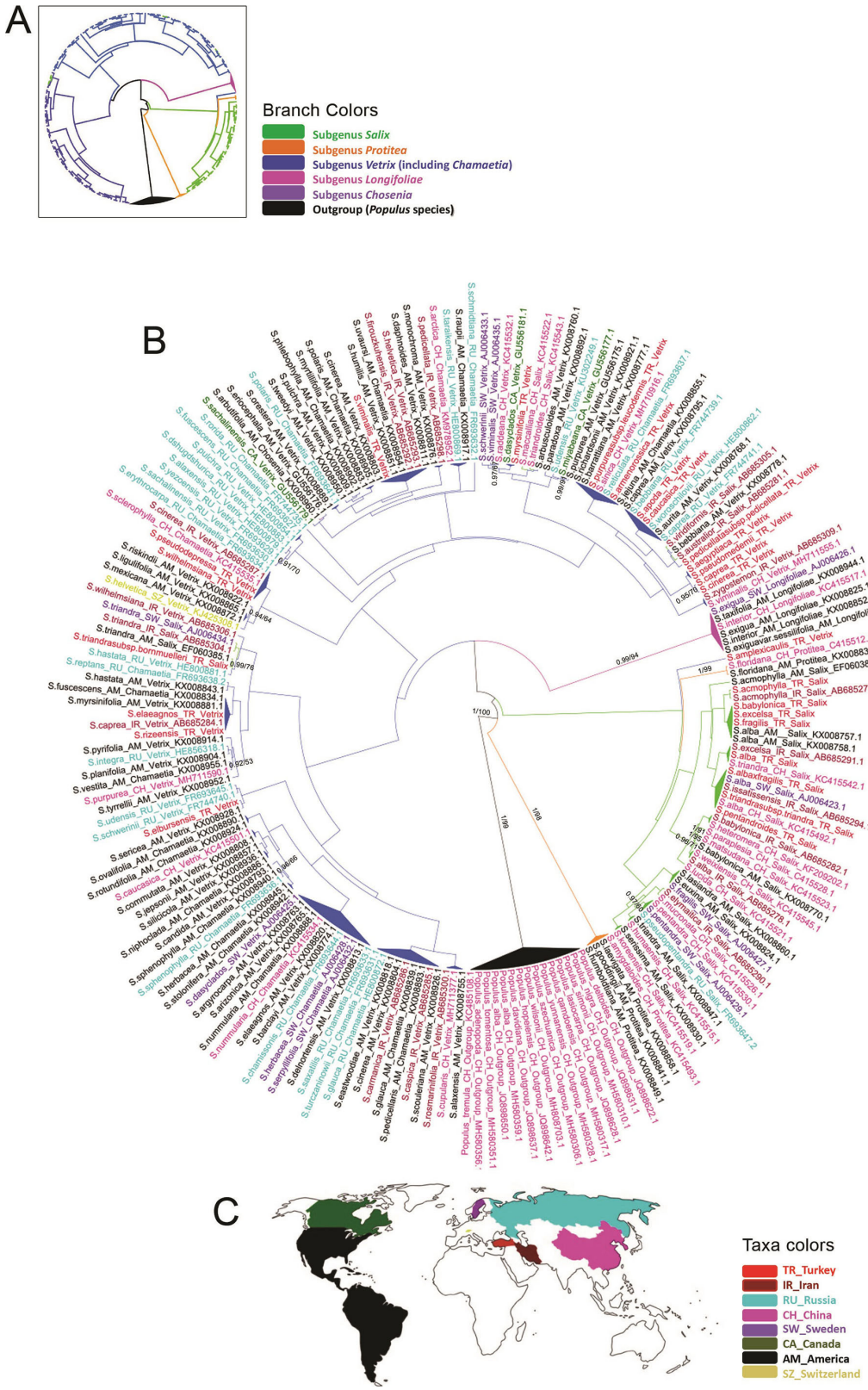

Fig. 3. Beast nrDNA (ITS) phylogenetic tree constructed with world *Salix* sequences obtained from GenBank. The numbers on branch are posterior probability from the Bayesian inference and the bootstrap values from the maximum parsimony, respectively. The Country abbreviation, Subgenera, GenBank Accession number are indicated behind the species name. (A) Phylogenetic relationships of *Salix* subgenera. The color in branch and legend represent the subgenera. (B) A detailed phylogeny of *Salix* species from the world. (C) A map showing the color of country to which species belong in the world. Bayesian posterior probabilities (up to 1) and bootstrap values (up to 100) are shown beside branches (pp/ bs) where posterior probability ≥0.90 or bootstrap value >50

other hand, ITS tree topology showed five major groups as subg. *Salix* s. str., *Vetrix, Chamaetia*, *Longifoliae*, and *Protitea* shown in different branch colors (Fig. 3). Most of the subg. *Protitea* members formed a well-supported monophyletic group with high posterior probability and bootstrap values (1/98) whereas some of them were nested in subg. *Salix* s. str (orange branch color). A group with blue branch color includes all species of subgenera *Chamaetia* and *Vetrix*. Subg. *Longifoliae* members (pink branch color) were clustered with subgenera *Vetrix* and *Chamaetia*. The limited number of individuals (*S. arbutifolia*) is insufficient to interpret the *Chosenia* group.

The phylogenetic tree constructed using the data from the chloroplast sequences (*trn T-F, matK,* and *rbcL*) supported two major groups with high posterior probability and bootstrap values as 1/100 (Fig. 2a). The first major group that mostly including species from subg. *Vetrix* had three clades (Fig. 2b). *S. elaeagnos* Scop. (all samples from Kastamonu province), *S. elbursensis* Boiss. (from Artvin), and *S. purpurea* subsp. *leucodermis* (from Muğla) were closely related and grouped under Clade 1. The Clade 2 consisted of two subclades. Some members of the section *Vetrix* such as *S. caprea* L., *S. caucasica* Andersson, and *S. pedicellata* subsp. *pedicellata* Desf. and one member of the section Hastatae, that is *S. apoda* Trautv. were submerged under one subclade. The branch values in the representatives of species from different locations were resulted in low posterior probability and bootstrap values compared with the samples of the Clade 1. The other subclade of the Clade 2 included *S. pseudomedemii* E. Wolf, *S. armenorossica* A. Skv. (all from Erzurum), and *S. cinerea* L. The last clade of subg. *Vetrix*, the Clade 3 consisted of *S. myrsinifolia* Salisb. (all from Kastamonu) and *S. aegyptiaca* L. The sister groups which are distantly located among the members of the subg. *Vetrix* were *S. pseudodepressa* A. Skv, *S. viminalis* L. (all from Erzurum) and *S. wilhelmsiana* Bieb. The second major group of genus *Salix* (The Clades 4 & 5) in the cpDNA tree, subg. *Salix* which was diverged from subg. *Vetrix* with significant posterior probability and bootstrap values (1/91) included mostly members of the section *Salix*. The Clade 4 consisted of only *S. babylonica* L. and *S. pentandroides* A. Skv. Interestingly, samples of *S. amplexicaulis* Bory and Chaub and *S. rizeensis* which were reported previously as the members of subg. *Vetrix*, were located as sister clades with very high posterior probability and bootstrap values among the subg. *Salix* group (1/100). The Clade 5 had two subclades. One of them was with *S. alba* L., *S. excelsa* J.F. Gmelin, *S. fragilis* L., and *S. alba x fragilis* which had wide distribution range in Turkey. The second subclade of the Clade 5 consisted of *S. triandra* subsp. *triandra* L., *S. triandra* subsp. *bornmuelleri* (Hausskn.) A. Skv., and *S. acmophylla* Boiss. Samples of *S.acmophylla* collected from southeastern Turkey. As expected, *S. alba x fragilis* morphologically identified as hybrid species was very closely associated with *S. alba* (nrDNA) and *S. fragilis* (cpDNA).

The results from the analysis of ITS sequence data revealed the major groups of subg. *Salix* s. str., *Vetrix, Chamaetia, Longifoliae*, and *Protitea* with high posterior probability and bootstrap values (1/100) (Fig. 3a). The closest relatives of *Salix* species of Turkey were determined and shown in Fig. 3. The first major group of the ITS phylogenetic *Salix* tree was the subgenus *Vetrix* (including *Chamaetia*) which involved some clades diverged from other subgenera with high number of taxa (Fig. 3b). One of the Clade of *Vetrix* (including *Chamaetia*) was comprised of the closely related species of Turkey (_TR) namely *S. pedicellata* subsp. *pedicellata*, *S. aegyptiaca*, *S. pseudomedemii*, *S. apoda*, *S. purpurea* subsp. *leucodermis* (endemic to Turkey) and *S. armenorossica*. *S. rizeensis* (endemic to Turkey) and *S. pseudodepressa* were located among American willows. *S. caprea* and *S. cinerea* were very closed to each other and grouped under one of the Clades of subgenus *Vetrix* (0.95/70). Exceptional subg. *Salix* s. str. members located in major group of subg. *Vetrix* (including *Chamaetia*) were *S. triandra* subsp. *bornmuelleri* (_TR), *S. triandra* (_AM, _SW, _IR), *S. triandroides* (_CH), *S. maccaliana* (_CH), *S. australior* (_IR) and *S.viridiformis* (_IR). The *S. triandra* subsp. *bornmuelleri* (_TR) was attached to that clade which involved the other samples of *S. triandra* (_ AM, _SW, _IR) with high posterior probability and bootstrap values (0.99/76). Subg. *Longifoliae* members (*S. exigua* (_SW, _AM), *S. exigua* var. *sessilifolia* (_AM), *S. interior* (_CH, _AM), and *S. taxifolia* (_AM)) formed a distinct subclade in ITS tree (0.99/94). The closely related species of subg. *Salix* s. str. were *S. paraplesia* (1/91), *S. matsudana* (1/95), *S. weixiensis* (0.96/71) and *S. lucida* from China. *S. acmophylla* (_TR, IR, AM), *S. babylonica* (_TR), *S. excelsa* (_TR) and *S. fragilis* (_TR) were grouped with *S. floridana*_CH and _AM under the same Clade of subg. *Salix* s. str.

The molecular dating analyses showed the diversification of subgenera *Salix* and *Vetrix* from the *Populus* species occurred about 46.92 Mya (Fig. 2b). The estimated divergence time of subg. *Salix* and subg. *Vetrix* dated back to 23.1 and 21.65 Mya, respectively. The diversification time for two subgenera species in Turkey was found relatively recent (Pliocene). The most recent diverging species seemed to be *S. viminalis, S. pseudodepressa, S. myrsinifolia, S. apoda, S. pedicellata* subsp. *pedicellata* and *S. rizeensis* (about 0.36 Mya).

Discussion

Phylogenetic implications

Previous studies have reported that the non-coding regions of chloroplast genome such as *trn T-F* usually have a high potential for mutation (Taberlet et al., 1991; Bakker et al., 2000; Hamza-Babiker et al., 2009) as found in *Salix* species of Turkey. The high polymorphism observed in *matK* is consistent with the findings of Percy et al. (2014) for the North American *Salix* species. The occurrence of highly variable sites can be explained by the long-aligned sequence of *matK* including some intron regions of *trn K*. Nevertheless, the conserved 3' end of *matK* with a high number of informative sites was very useful in resolving taxonomic problems. However, the *rbcL* region was highly conserved in the studied *Salix* taxa. This gene had a low resolving power to address the problems of lower taxonomic levels in the genus. Thus, the sequences of uniparentally inherited cpDNA except for the *rbcL*, have provided sufficient information to comprehend interspecific relations of *Salix* subgenera (*Salix* and *Vetrix*).

The results of the current study from cpDNA data are in accordance with the classification system of Skvortsov (1999) in which the *Salix* species of Turkey can be classified into two, namely subg. *Salix* and *Vetrix*. Similar clade formations have also been reported for the Japanese (Azuma et al., 2000), Chinese (Chen et al., 2010; Zhao et al., 2019) and American *Salix* species in respected subgenera (Lauren-Moreau et al., 2015). However, the species of subgenera *Chamaetia* and *Vetrix* ended up in the same group with moderately high posterior probability and bootstrap values (0.87/76) in the ITS phylogenetic tree of the current study. This result including subg. *Vetrix* species from Turkey is still consistent with previous studies (Azuma et al., 2000; Chen et al., 2010, Wagner et al., 2018), supporting a merge of the two *Salix* subgenera (*Chamaetia* and *Vetrix*) under a single subgenus. Besides, different from other studies (Abdollahzadeh et al., 2011; Lauren-Moreau et al., 2015; Wu et al., 2015), subg. *Longifoliae* was clustered with subg. *Vetrix* (including *Chamaetia*) members, rather than nesting in subg. *Salix*. Also, the ITS sequences of subg. *Protitea* (from America and China) resulted in a monophyletic major clade except for two accessions of *S. floridana* which are roughly related to subgenera. Therefore, unlike other subgenera system, we suggest the division of *Salix* L. into four subgenera, *Salix* s. str. and *Vetrix* (including *Chamaetia*), *Protitea*, and *Longifoliae* for the infrageneric system of *Salix* in the world.

The members of subg. *Salix* Section *Amygdalinae* W.Koch including *S. triandra* subsp. *bornmuelleri* (_TR), *S. triandra* (_AM, _SW, _IR) and *S. triandroides* (_CH) were located in the subg. *Vetrix* (including *Chamaetia*) group of the ITS *Salix* phylogeny. Since members of this section diverged from the monophyletic nature of the subg. *Salix* (Chen et al., 2010; Abdollhazadeh et al., 2011), *Salix triandra* with different ploidy level (2x=2n=38) is always chosen as an outgroup in *Salix* phylogeny studies (Wagner et al., 2018; Wagner et al., 2020). Distant position of *S. triandra* was also supported by morphological traits such as life form, bark type, stipule persistence, leaf shape, twig slender, decorticated wood, bud angle and petiole length (Acar et al., 2020). Even if subspecies of *Salix triandra* were clustered under subg. *Salix* in our cpDNA tree, the exclusion of Section *Amygdalinae* W.Koch members from subg. *Salix* (Wu et al., 2015) was also supported by the ITS data from the current study and morphological data from Acar et al. (2020). As far as both cpDNA and nrDNA based trees are concerned, the most distant species of subg. *Vetrix* were *S. wilhelmsiana* and *S. pseudodepressa*. The distant position of *S. pseudodepressa* can be explained by the phenomena: the rare nature of the species due to its completely isolated natural occurrences in high altitude habitats (Acar & Usta Baykal, 2020).

Introgressive hybridization and incomplete lineage sorting are generally considered as the sources of phylogenetic contradictory between topology of cpDNA and nrDNA trees in willows (Wayne & Knowles, 2006; Stegemann et al., 2012). It is difficult to identify incomplete lineage sorting or gene flow as a result of interspecific hybridization with the current type of data set. However, hybridization is known to be common among biogeographically close and locally distributed *Salix* species (Stegemann et al., 2012; Percy et al., 2014). Specifically, subg. *Salix* species prefers habitats with continental climates such as Central Anatolia (*S. fragilis*) and Southeastern Turkey (*S. acmophylla*), whereas the subg. *Vetrix* species (*S. elaeagnos, S. elbursensis, S. apoda, S. myrsinifolia, S. caucasica,* and *S. rizeensis*) are found in and adapted to wet and cool climates of the high latitude and altitude habitats of northern and eastern Turkey (Acar & Usta Baykal, 2020). Diverse climate and habitats existing in Turkey may facilitate the lateral haplotype transfer (Stegemann et al., 2012) in closely related willow species wherever they share mixed habitats.

Biogeographical implications

The clear separation of two subgenera of Turkish *Salix* species may result from different biogeographic barriers such as Anatolian Diagonal and major mountain ranges in southern and northern Turkey. The morphological study on *Salix* from Turkey confirmed that subg. *Salix* is a natural group displaying with distinct morphological characteristics such as tree-like life forms and lanceolate leaf shapes, while subg.

Vetrix includes species characterized by evolutionary advanced traits (pubescence on bud scale) (Acar et al., 2020). The subg. *Salix* section *Salix* members including *S. alba*, *S. fragilis*, *S. alba x fragilis*, and *S. excelsa* with many ancestral characters are found as closely related in phylogenetic trees of the current study. Therefore, it is reasonable to assume that the section was dispersed and adopted to the warm-temperature regions of the world (Skvortsov, 1999). The complex relation and extensive polytomy of subg. *Vetrix* have been observed in the current study as reported by several previous studies (Leskinen & Alström-Rapaport, 1999; Hardig et al., 2010; Abdollahazadeh et al., 2011; Barkalov & Kozyrenko, 2014). The observed relationships among subg. *Vetrix* species could be explained by natural hybridization events since hybridizations are still continued within the geographically close members of the subgenus which are mainly originated in the northern latitudes.

The taxonomically well-defined and geographically distinct *Salix* species from the world were involved clades in which they are genetically similar in ITS tree. All twenty-six *Salix* taxa native to Turkey scattered throughout the world *Salix* accessions (158) and aligned with related subgenera level positions in the analysis. Thus, the close molecular relationships between *Salix* species of Turkey and *Salix* species from the world seem to be determined by taxonomical affinities, rather than distinct geographical distribution. Members of subg. *Vetrix* native to Turkey have close relations with American willows from subg. *Vetrix* supporting statement that the migration way from Asia to North America caused *Salix* movement and rapid diversification in subg. *Vetrix* members (Wu et al., 2015; Özdilek et al., 2012).

Three *S. acmophylla*_TR, _IR, _AM samples were well allied, but it was the furthest clade of subg. *Salix* in the ITS *Salix* phylogeny. The similar placement of *S. acmophylla* was reported and supported by morphological dataset (Hardig et al., 2010; Acar et al., 2020). Contrary to the ITS tree, the position of *S. acmophylla* was under subg. *Salix* in cpDNA tree. The explanation for this may be due to the hybrids occurring between *S. alba* and *S. acmophylla* (Abdollahzadeh et al., 2011; Barkalov & Kozyrenko, 2014). The other scenario for *S. acmophylla*_TR is that the effect of the Anatolian Diagonal may have caused to speciation due to isolation mechanism created by natural mountain barriers (Bilgin, 2011). The species is likely to be evolved in generally continental climates of eastern Irano–Turanian phytogeographic region of Anatolia.

Molecular dating

Two main clades of genus *Salix* in Turkey shared the same biogeography were very similar according to divergence time based on combined cpDNA data (Oligocene). There are many fossil records from the North America (Wolfe, 1987), Europe and Russia (Collinson, 1992) that estimated Eocene and Oligocene origin of genus *Salix*. Wu et al. (2015) obtained the crown group age of 23.76 Mya (subg. *Vetrix*) which is comparable to our findings. The origin and split dating of the *Salix* subgenera of Turkey went back to Eocene (45.1 Mya), but the occurrence time of most of the species of genus *Salix* indigenous to Turkey were in Pliocene. Especially, members of subg. *Vetrix* undergone a recent diversification in high altitude of Anatolia (before 5 Mya). This dating has been supported by the findings of Kasaplıgil (1977) who reported the age of Anatolian *Salix* fossil record dated back to the Pliocene (2.58–5.33Mya). Thus, the last glacial period acting as glacial cover for forest biodiversity (starting from late Pliocene: 2.58 Mya) has an effect on shaping the current composition of *Salix* species in Turkey (Ledig, 1998).

The new findings on the two (cpDNA data) and five (nrDNA data) subgenera of *Salix*. L. were summarized in the current study. Although *S. amplexicaulis* (cpDNA and nrDNA) and *S. rizeensis* (cpDNA) are taxonomically considered the members of subg. *Vetrix,* the results from the current molecular data suggest that these species are phylogenetically close to subg. *Salix*. The morphological affinity for *S.amplexicaulis* and *S.rizeensis* (both possess glabrous bud scale) supports the recognized phylogenetic positions of both species in subgenus *Salix* (Acar et al., 2020). The positions of *S. rizeensis* and *S. amplexicaulis* in subg. *Vetrix* instead of in subg. *Salix* could be due to natural hybridization occurring between different *Salix* species of Anatolia in a species mixed zone such as Black Sea and Central Anatolia Regions (Supplemantary Table S1). The results of the current study clearly helped to determine the phylogenetic positions of 11 *Salix* taxa in Turkey (*S. pentandroides*, *S. apoda*, *S. armenorossica*, *S. pseudomedemii*, *S. pedicellata* subsp. *pedicellata*, *S. pseudodepressa*, *S. amplexicaulis*, *S. purpurea* subsp. *leucodermis*, *S. rizeensis*, *S. triandra* subsp. *triandra* and *S.triandra* subsp. *bornmuelleri*) among the world *Salix* species for the first time. Those new records will also bring new sequence data in Genbank. The findings are important not only for providing new chloroplast and nuclear genome sequence data for future phylogenetic and evolutionary studies, but also for exploring economically valuable species and relatives such as *S. amplexicaulis* with respect to obtaining medicinal products (Alakkarı, 2017; Akyürek & Acar, 2020).

Conclusion

The study explored phylogenetic placement of *Salix* taxa native to Turkey with large number of samples

based on a combined data sets from cpDNA and nrDNA sequences (4550 bp) for the first time. The constructed phylogenetic tree of the *Salix* species found in Turkey showed a monophyly and supported well two subgenera (*Salix* and *Vetrix*). While two coding cpDNA sequences (*matK* and *rbcL*) were found to be somewhat conserved, the non-coding cpDNA (*trn T-F*) and nrDNA ITS regions had a high number of variable sites in *Salix* species. The distant position of *S. acmophylla* (among species of subgenus *Salix*), integration of *Chamaetia/Vetrix* subgenera and removal of Section *Amygdalinae* W.Koch members from subg. *Salix* were also strongly supported by our findings. All investigated *Salix* species have received their taxonomic position of subgenera in ITS *Salix* phylogenetic tree regardless of geographical origin. The crown age of two subgenera from Turkey was found to be at the Eocene. Subgenus *Salix* and subg. *Vetrix* diversification dated back to the Oligocene. The most recently diverged species were found in subg. *Vetrix* (0.36 Mya). We firmly revealed the phylogenetic positions of 11 *Salix* taxa with a large amount of data at first time in the world and proposed that phylogenetic positions of *S.amplexicaulis* and *S.rizeensis* (endemic to Turkey) species are closer to subg. *Salix* rather than subg. *Vetrix*. We believe that the new chloroplast and nuclear genome related DNA sequence data and findings from the current study will greatly contribute to the future *Salix* phylogenetic and evolution related studies.

Conflicts of interest statement

The authors declare no potential conflict of interest.

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