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Phylogeny of Anatolian (Turkey) species in the *Digitalis* sect. *Globiflorae* (Plantaginaceae)

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Abstract

This study analyses phylogenetic and morphological relationships regarding the taxonomy of *Digitalis* sect. *Globiflorae*. Progesterone 5β-reductase (P5βR) is an important enzyme for cardenolide biosynthesis as well as a novel genetic marker inferring phylogenetic and biogeographic relationships in many plant species. Phylogenetic inference was conducted using the neighbor-joining method and maximum likelihood model on cDNA sequences of highly conserved P5βR (97.0–99.6%) isolated from members of sect. *Globiflorae*, including *Digitalis trojana*, *D. cariensis*, *D. lamarckii, D. lanata* subsp. *lanata*, *D. ferruginea* subsp. *ferruginea* and *D. ferruginea* subsp. *schischkinii*. Our phylogenetic and morphological results largely support Davis' system of the genus *Digitalis* in the *Flora of Turkey*. We propose the existence of four species and three subspecies in sect. *Globiflorae* for Turkey. *Digitalis cariensis* is a distinct species endemic to Turkey. In spite of high morphological similarities, *D. lamarckii* was separated from *D. trojana* and *D. lanata*. *Digitalis trojana* is accepted at the infraspecific rank as *D. lanata* subsp. *trojana*. In addition, *D. ferruginea* is ranked under the two subspecies following Werner's treatment. Our study presents a new understanding of the speciation patterns of endemic and non-endemic eastern *Digitalis* species distributed in Anatolia, Turkey, underlying molecular, biogeographical and comparative morphological analyses.

Key words: Anatolia, *Digitalis*, Morphology, Phylogeny, Progesterone 5β-reductase (P5βR), section *Globiflorae*, Taxonomy

Introduction

Following Linnaeus, who categorized *Digitalis purpurea* Linnaeus (1753: 621), several researchers have attempted to deduce the taxonomy of *Digitalis* Linnaeus (1753: 621) species, commonly known as foxgloves (Lindley 1821, Ivanina 1955, Werner 1960, 1964, 1965, Heywood 1972, Davis 1978, Bräuchler *et al*. 2004, Herl *et al.* 2008, Kelly & Culham 2008). Of these, Werner (1960, 1964, 1965) established well-accepted taxonomy recognizing 19 species confined to continental Europe, the British Isles, Madeira, North Africa, Anatolia and the Caucasian part of Asia. Additionally, Heywood (1972) and Meusel *et al.* (1978) followed Werner's description based on phytogeographical and morphological characters (Table 1). After the disintegration of Scrophulariaceae (Olmstead *et al.* 2001), the genus *Digitalis* was treated as a member of Plantaginaceae (Albach *et al.* 2005).

Werner (1965) regarded the Eastern and Western Mediterranean area as the major diversity centre of *Digitalis*. As for the eastern *Digitalis*, there are some incongruities between Werner's and Davis' systematic treatments in terms of nomenclature of some endemics growing in Turkey. In the *Flora of Turkey and the East Aegean Islands*, Davis (1978) stated that the genus *Digitalis* is represented by eight species and two subspecies. Herein, endemic species including *Digitalis cariensis* Boissier ex Bentham (1846: 450), *D. lamarckii* Ivanina (1955: 260) and *D. trojana* Ivanina (1955: 263) do not fit the subspecies level of "*cariensis alliance*" proposed by Werner (1960, 1964), who placed *Digitalis lamarckii* and *D. trojana* under *D. cariensis* as infraspecific taxa. Instead, the "*cariensis alliance*" is placed at species level in Davis' system (as mentioned throughout this paper). To the best of our knowledge, there have been no arguments

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proposed by any taxonomist in order to interpret this discrepancy either in later volumes of the *Flora of Turkey* or in any individual taxonomical studies as a kind of revision for Anatolian *Digitalis* species. Moreover, investigation of *Digitalis* taxa in North Anatolia might provide a new understanding of sect. *Globiflorae* Bentham (1846: 450) as Bräuchler *et al.*(2004) stated the complexity of this section might be reduced with new samplings.

TABLE 1. Systematic classification of the genus *Digitalis* proposed by Werner (1965) to Herl *et al.* (2008).

Species Name	**"Strict consensus" (ITS; trnL-F; P5βR) Herl *et al.* (2008)**	**Bräuchler *et al.* (2004)**	**Werner (1965) Heywood (1972)**
D. minor Linnaeus (1771: 567) *D. purpurea* Linnaeus (1753: 621) *D. thapsi* Linnaeus (1763: 867) *D. mariana* Boiss. (1841: 465)	Sect. *Digitalis* Linnaeus (1753: 621)	Sect. *Digitalis*	Sect. *Digitalis*
D. atlantica Pomel (1875: 300) *D. ciliate* Trautvetter (1866: 397) *D. davisiana* Heywood (1949: 164) *D. grandiflora* Miller (1768: 4) *D. viridiflora* Lindley (1821: 21)	No material Sect. *Macranthae* Heywood (1972: 357)	Sect. *Macranthae*	Sect. *Grandiflorae* Bentham (1846: 450) emend. Werner (1960: 230) *Tubilorae*
D. sceptrum Linnaeus (1782: 282) = *Isoplexis sceptrum* (Linnaeus, 1782: 282) Loudon (1829: 528) *D. canariensis* Linnaeus (1753: 621) = *I. canariensis* (L.) Loudon (1829:528) *D. chalcantha* (Sventenius & O'Shanahan) Albach, Bräuchler & Heubl (2008: 76) = *I. chalcantha* Sventenius & O'Shanahan (1969: 47) *D. isabelliana* (Webb & Berthelot, 1845: 143) Lindinger (1926: 130) = *I. Isabelliana* (Webb & Berthelot) Morris (1896: 67)	Sect. *Isoplexis* Lindley (1821: 2)	Sect. *Isoplexis*	Genus *Isoplexis* (Lindley, 1821: 2) Bentham (1835: 1770)
D. ferruginea subsp. *ferruginea* Linnaeus (1753: 622) *D. lanata* subsp. *lanata* Ehrhart (1792: 152) *D. laevigata* Waldstein & Kitaibel (1803-1804: 171) *D. nervosa* Steudel & Hochstetter ex Bentham (1846: 450)	Sect. *Globiflorae* Bentham (1846: 450)	Sect. *Globiflorae*	Sect. *Globiflorae*
D. lanata Ehrhart (1792: 152) subsp. *leucophaea* (Smith, 1809: 439) Werner (1960: 242)	No data		
D. lanata Ehrhart (1792: 152) subsp. *trojana* (Ivanina, 1955: 263) Yücesan & Eker *stat. nov.*	No data		
D. cariensis Boissier ex Bentham (1846: 450)	No data		
D. lamarckii Ivanina (1955: 260)	No data	No data	
D. ferruginea Linnaeus (1753: 622) subsp. *schischkinii* (Ivanina, 1946: 204) Werner (1960: 238)	No data	No data	
D. obscura Linnaeus (1763: 867)	Sect. *Frutescentes* Bentham (1846: 452)	Sect. *Frutescentes*	Sect. *Frutescentes*
D. parviflora Jacquin (1770: 6)	Not clear	Sect. *Parviflorae* informally named	Sect. *Tubiflorae* Bentham (1846: 452)
D. subalpine Braun-Blanquet (1928: 345)	Not clear	Sect. *Subalpinae* informally named	
D. lutea Linnaeus (1753: 622)	Sect. *Macranthae*	Sect. *Macranthae*	
D. × *sibirica* (Lindley, 1821: 16) Werner (1960: 249)		Hybrid of *D. grandiflora* and *D. laevigata*	

Several *Digitalis* species are potent sources of cardiac glycosides that regulate heart rhythm. Of these, especially *D. lanata* Ehrhart (1792:152) and *D. purpurea* have been evaluated as major cardiac glycoside sources for drug producers (Mohammed *et al.* 2015). Technical improvements in molecular science are attractive to researchers who attempt to discover new insights through taxonomy of medicinal plants, and understanding of the biosynthesis of natural compounds. Thus, a detailed discussion concerning the molecular phylogeny of the genus *Digitalis* was published by

Bräuchler *et al.* (2004) and Herl *et al.* (2008). Using morphological, biogeographical and molecular phylogeny data, taxonomical investigations of varying extent were reported over the last two decades (Carvalho & Culham 1998, Nebauer *et al.* 2000, Kelly & Culham 2008). Herl *et al.* (2008) investigated a phylogenetic relationship within the genus *Digitalis* including several *Isoplexis* (Lindley 1821: 27) Loudon (1829: 528) species employing the sequence of the progesterone 5β-reductase (*P5βR*) gene. *P5βR* belongs to the VEP1 gene family and encodes for a progesterone 5β-reductase. *P5βR* catalyses the reduction of progesterone into 5β-pregnane-3,20-dione. Thus, it is considered a key enzyme in the biosynthesis of 5β-cardenolides (Gärtner & Seitz 1993, Kreis *et al.* 1998). The gene was also identified (*Dop5βr*) in the Spanish endemic *D. obscura* Linnaeus (1763: 867) (Roca-Perez *et al.* 2004) and other *Digitalis* species (incl. *Isoplexis*) (Herl *et al.* 2008). *P5βR* genes are highly conserved not only in the genus *Digitalis* but also in other angiosperms and their occurrence is not limited to 5β-cardenolide-forming plant species (Bauer *et al.* 2010, Munkert *et al.* 2011). P5βR may hence be used as an alternative molecular marker in addition to the well-known ITS and *trnL-F* sequences (Herl *et al.* 2008).

Bräuchler *et al.* (2004) and Herl *et al.* (2008) pointed out the inadequacy of data with respect to speciation patterns of eastern *Digitalis* species growing in Anatolia. Moreover, some discrepancies in nomenclature between Werner (1960, 1964) and Davis (1978) are discussed in the present paper. Therefore, the main objectives of the present study are: (1) to extrapolate the phylogeny of Anatolian foxgloves collected from natural populations of *Digitalis* species in sect. *Globiflorae* (*D. lanata* subsp. *lanata*, *D. cariensis*, *D. trojana*, *D. lamarckii*, *D. ferruginea* Linnaeus (1753: 622) subsp. *ferruginea* and *D. ferruginea* subsp. *schischkinii* (Ivanina, 1946: 204) Werner (1960: 238)) using their P5βR gene sequences as a genetic marker; (2) to compare morphological characters of collected taxa with those of previous studies and to understand the biogeographical distribution of taxa in order to make an inference about the current nomenclature of the species.

Materials and Methods

Plant material

Both flowering and fruiting specimens of *Digitalis* species were collected from their natural habitats in Turkey, and dried according to standard herbarium protocols (Table 2; Figs. 1–6). Voucher specimens are deposited in the herbarium of Abant İzzet Baysal University (AIBU). Molecular experiments were initiated after germination of the respective seeds under greenhouse conditions. Young leaves (four to eight weeks old) were used for molecular biology studies.

TABLE 2. Details of selected samples analyzed in the morphological studies

Voucher/ Source	Date	Taxon	Locality/Origin	Altitude	Coordinates
Eker 1727	15.9.2006	*D. cariensis*	Alanya/Antalya	981 m	N 36° 30.951' / E 032° 12.852'
EGE 5358	9.7.1966	*D. cariensis*	Fethiye/Muğla	1000 m	–
EGE 5014	12.7.1966	*D. cariensis*	Köyceğiz/Muğla	1200 m	–
EGE 4963	17.6.1967	*D. cariensis*	Nif Köyü/Muğla	–	–
EGE 8074	25.6.1969	*D. cariensis*	Anamas/Isparta	–	–
EGE 7393	9.7.1984	*D. cariensis*	Çal Dağı/Muğla	1900–2000 m	N 36° 50' / E 29° 08'
EGE 19433	12.6.1996	*D. cariensis*	Tire/İzmir	500–800 m	–
EGE 18950	6.9.1994	*D. cariensis*	Honaz/Denizli	1700 m	–
Eker 1726	12.9.2006	*D. lamarckii*	Mudurnu/Bolu	1095 m	N 40° 25.496' / E 031° 09.842'
Eker 1973	20.10.2007	*D. lamarckii*	Çamlıdere/Ankara	1456 m	N 40° 37.709' / E 032° 26.265'
Eker 2142	9.5.2008	*D. lamarckii*	Zigana/Gümüşhane	1668 m	N 40° 37.349' / E 039° 22.877'
Eker 2162	11.5.2008	*D. lamarckii*	Akdağ/Amasya	1560 m	N 40° 46.039' / E 035° 52.877'
Eker 2611	2.6.2010	*D. lamarckii*	Beyşehir/Konya	1353 m	N 37° 27.894' / E 031° 33.808'
Eker 2946	19.6.2012	*D. lamarckii*	Kıbrıscık/Bolu	1092 m	N 40° 24.237' / E 031° 56.892'
Eker 3075	27.9.2012	*D. lamarckii*	Mengen/Bolu	588 m	N 40° 59.926' / E 031° 57.324'
Eker 3080	13.10.2012	*D. lamarckii*	Aladağlar/Bolu	1342 m	N 40° 30.650' / E 031° 42.950'

...Continued on next page

TABLE 2. (Continued)

Voucher/ Source	Date	Taxon	Locality/Origin	Altitude	Coordinates
Eker 5288	15.6.2014	*D. lamarckii*	Kazan/Ankara	957 m	UTM 480528 / 4452632
Eker 5368	12.7.2014	*D. lamarckii*	Kızılcahamam/Ankara	1395 m	UTM 467963 / 4476878
Eker 5394	19.7.2014	*D. lamarckii*	Beypazarı/Ankara	829 m	UTM 394794 / 4449985
Eker 5439	20.7.2014	*D. lamarckii*	Elmadağ/Ankara	1757 m	UTM 498059 / 4405581
Eker 1730	16.9.2006	*D. trojana*	Ida (Kazdağı)/Balıkesir	500 m	–
Eker 1905	8.6.2007	*D. trojana*	Ida (Kazdağı)/Balıkesir	250 m	N 39° 38.615' / E 026° 57.552'
Eker 1906	8.6.2007	*D. trojana*	Ida (Kazdağı)/Balıkesir	309 m	N 39° 38.751' / E 026° 57.450'
Eker 2435	30.5.2009	*D. trojana*	Ida (Kazdağı)/Balıkesir	435 m	N 39° 39.650' / E 026° 57.578'
Eker 3455	18.6.2013	*D. lanata*	Eceabat/Çanakkale	24 m	N 40° 10.11' / E 036° 22.26'
Eker 3460	19.6.2013	*D. lanata*	Dereköy/Kırklareli	493 m	N 41° 51.89' / E 027° 18.89'
Eker 3474	21.6.2013	*D. lanata*	Ganos/Tekirdağ	120 m	N 40° 51.38' / E 027° 27.35'
Eker 3628	24.7.2013	*D. lanata*	Armağanköy/Kırklareli	523 m	N 41° 53.991' / E 027° 23.832'
Eker 3637	24.7.2013	*D. lanata*	Kıyıköy/Kırklareli	203 m	N 41° 39.666' / E 027° 55.802'
Eker 3641	26.7.2013	*D. ferruginea* subsp. *schischkinii*	Ilgaz Dağı/Kastamonu	1628 m	N 41° 02.815' / E 033° 44.502'
Eker 3643	26.7.2013	*D. ferruginea* subsp. *schischkinii*	Küre Dağları/Sinop	753 m	N 41° 41.445' / E 034° 54.446'
Eker 3644	27.7.2013	*D. ferruginea* subsp. *schischkinii*	Ünye/Ordu	317 m	N 41° 03.385' / E 037° 20.142'
Eker 3645	27.7.2013	*D. ferruginea* subsp. *schischkinii*	Çambaşı/Ordu	1576 m	N 40° 43.538' / E 037° 56.437'
Eker 3646	27.7.2013	*D. ferruginea* subsp. *schischkinii*	Tamdere/Giresun	1351 m	N 40° 32.329' / E 038° 21.475'
Eker 3647	28.7.2013	*D. ferruginea* subsp. *schischkinii*	Sis Dağı/Trabzon	1749 m	N 40° 51.260' / E 039° 09.367'
Eker 3651	28.7.2013	*D. ferruginea* subsp. *schischkinii*	Hamsiköy/Trabzon	1404 m	N 40° 41.542' / E 039° 27.834'
Eker 3659	29.7.2013	*D. ferruginea* subsp. *schischkinii*	Anzer/Rize	2157 m	N 40° 34.823' / E 040° 30.773'
Eker 3672	29.7.2013	*D. ferruginea* subsp. *schischkinii*	Cimil/Rize	1883 m	N 40° 44.298' / E 040° 44.927'
Eker 3674	30.7.2013	*D. ferruginea* subsp. *schischkinii*	Borçka/Artvin	149 m	N 40° 44.294' / E 040° 44.925'
Eker 3675	30.7.2013	*D. ferruginea* subsp. *schischkinii*	Maçahel/Artvin	855 m	N 41° 28.832' / E 041° 58.613'
Eker 2614	2.6.2010	*D. ferruginea* subsp. *ferruginea*	Beyşehir/Konya	1353 m	N 37° 27.894' / E 031° 33.808'
Eker 3055	24.9.2012	*D. ferruginea* subsp. *ferruginea*	Abant/Bolu	1337 m	N 40° 36.452' / E 031° 17.605'
Eker 3073	27.9.2012	*D. ferruginea* subsp. *ferruginea*	Yedigöller/Bolu	703 m	N 40° 56.960' / E 031° 44.876'
Eker 3074	27.9.2012	*D. ferruginea* subsp. *ferruginea*	Mengen/Bolu	588 m	N 40° 59.926' / E 031° 57.324'
Eker 3632	24.7.2013	*D. ferruginea* subsp. *ferruginea*	Şükrüpaşa/Kırklareli	643 m	N 41° 55.827' / E 027° 29.447'
Eker 3635	24.7.2013	*D. ferruginea* subsp. *ferruginea*	Balaban/Kırklareli	513 m	N 41° 47.138' / E 027° 42.091'
Eker 3636	24.7.2013	*D. ferruginea* subsp. *ferruginea*	Kıyıköy/Kırklareli	316 m	N 41° 36.116' / E 027° 50.254'
Eker 3638	25.7.2013	*D. ferruginea* subsp. *ferruginea*	Maşukiye/Kocaeli	398 m	N 40° 41.017' / E 030° 08.240'
Eker 3639	25.7.2013	*D. ferruginea* subsp. *ferruginea*	Kartepe/Kocaeli	1342 m	N 40° 38.504' / E 030° 06.826'
Eker 3640	25.7.2013	*D. ferruginea* subsp. *ferruginea*	Bolu Dağı/Bolu	890 m	N 40° 45.095' / E 031° 23.957'
Eker 3702	4.10.2013	*D. ferruginea* subsp. *ferruginea*	Sinop Burnu/Sinop	167 m	N 42° 01.904' / E 035° 11.542'
Eker 3704	4.10.2013	*D. ferruginea* subsp. *ferruginea*	Ayancık-Türkeli/Sinop	46 m	N 41° 53.897' / E 034° 34.497'
Eker 3705	4.10.2013	*D. ferruginea* subsp. *ferruginea*	Küre Dağları/ Kastamonu	502 m	N 41° 55.233' / E 033° 44.087'
Eker 4024	16.3.2014	*D. ferruginea* subsp. *ferruginea*	Nallıhan/Ankara	1365 m	UTM 341999 / 4456956

Morphological Analyses

Morphological descriptions of taxa based on measurements were examined in both fresh and dried samples of natural populations. All quantitative as well as most of the qualitative characters excluding the colour features were examined in dried specimens. Measurements were made using a precise ruler under a stereo-microscope. The *Flora of Turkey and the East Aegean Islands* (Davis, 1978) and the taxonomic revision of Werner (1960) were initially used for plant identification. The nomenclature of plant names was checked using *Türkiye Bitkileri Listesi* (Güner *et al.* 2012) as well as web sites, namely *International Plant Name Index* (IPNI 2014) and *The Plant List* (2014). Authors' names were

in accordance with Brummitt & Powell (1992) and IPNI (2014). In the morphological studies, non-discriminative characters given in previous studies among taxa were eliminated and discussed, while the main discriminative characters based on the new observations and measurements were given, and a new key for sect. *Globiflorae* is provided.

FIGURE 1. *Digitalis* species from Anatolia: *D. cariensis.* A. Habit, B. Inflorescence, C. Flower, D. Fruits (photographed by Eker).

FIGURE 2. *Digitalis* species from Anatolia: *D. lanata*. A. Habit, B. Inflorescence, C. Flower, D. Fruits (photographed by Eker).

RNA Isolation, Single Strand cDNA Production and PCR

Fresh leaf samples (*ca*. 200 mg) were ground to a fine powder using liquid nitrogen with a mortar and pestle. Total RNA isolation was carried out with an innu PREP Plant RNA kit (Analytik Jena AG, Jena, Germany). Single strand cDNA was synthesized by reverse polymerase chain reaction (RT-PCR) using a Super Script™ III RT-PCR kit according to the instructions provided by the manufacturer (Invitrogen, Karlsruhe, Germany). Purified total RNA up to 5 µg was used to synthesize cDNA. The amplification was carried out in a Personal Cycler 20 (Biometra GmbH, Göttingen,

Germany). Each PCR reaction (50 µL total volume) contained 2.5 units of PeqGold Taq DNA Polymerase (Peqlab GmbH, Erlangen, Germany), 10× reaction buffer S, 0.5 mM of dNTP mixture and 2 mM of appropriate primers (VH07spedir and VH1188salrev, Herl *et al.* 2006) and 2.0 µL of the respective cDNA. For the PCR amplification program, a method slightly modified from Bauer *et al.* (2010) was used as follows: initial denaturation at 95°C for 3 min was followed by 30 cycles of a 1-min denaturation period at 95°C, annealing at 63°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products were run on gel electrophoresis containing 1.0% agarose in a TAE buffer system. The size of the gene of interest was determined using Smart Ladder (Eurogentec GmbH, Köln, Germany), producing bands of different sizes ranging from 200 bp to 10,000 bp.

FIGURE 3. *Digitalis* species from Anatolia: *D. trojana.* A. Habit, B. Inflorescence, C. Flower, D. Fruits (photographed by Eker).

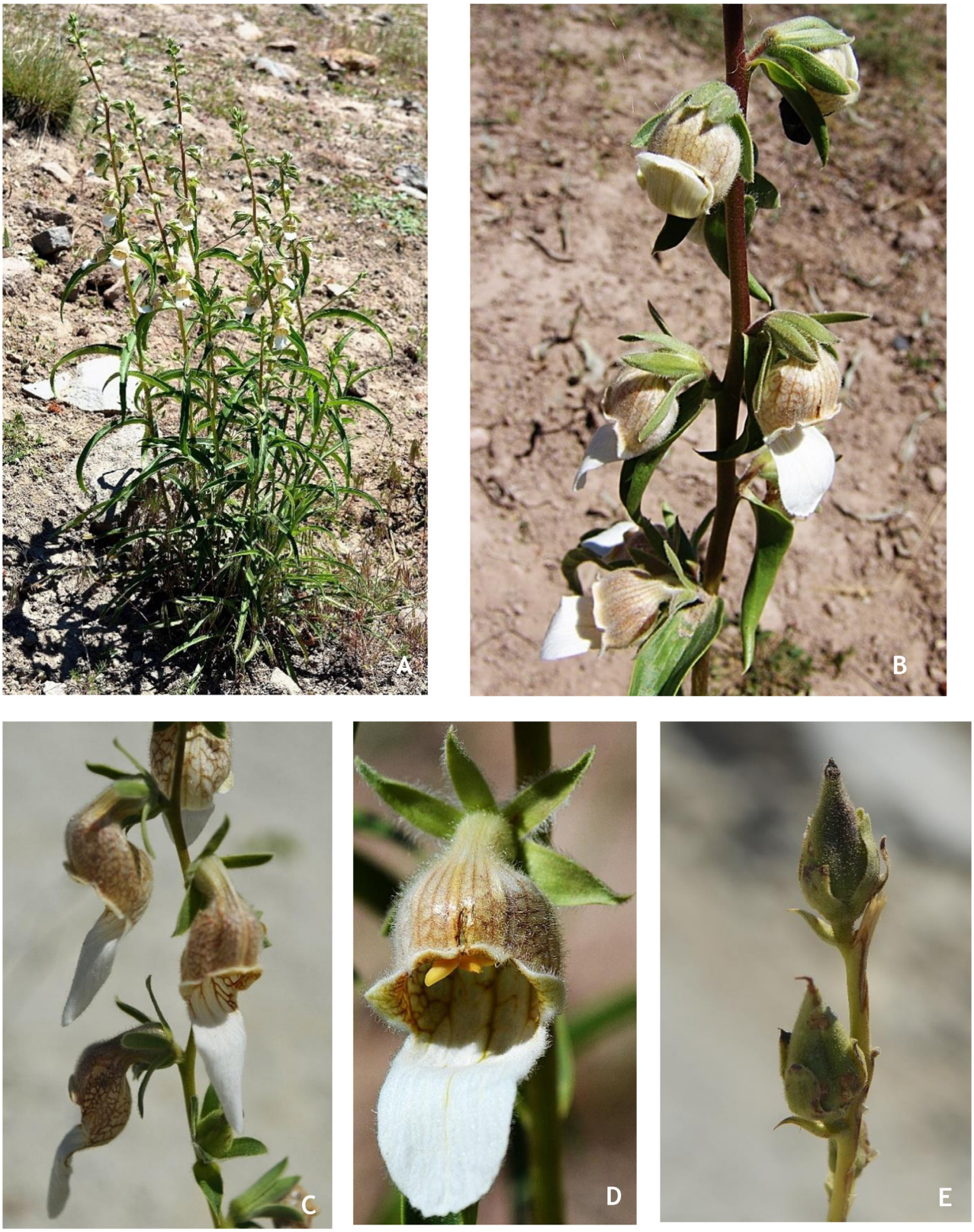

FIGURE 4. *Digitalis* species from Anatolia: *D. lamarckii.* A. Habit, B-C. Inflorescence, D. Flower, E. Fruits (photographed by Eker).

Ligation and Subcloning

After gene amplification confirmation, cloning was carried out as recommended by the TA cloning kit's manufacturer (Life Technologies, Carlsbad, CA, USA) with some modifications. Briefly, 3 µL of PCR product, 2 µL of 5× ligation buffer, 1 µL of plasmid pCR® 2.1 and 1 µL of express ligase were mixed. For successful ligation, the reaction was carried out at 16°C overnight. Ligated products were transformed into Top 10 *E. coli* competent cells at 42°C for

30 seconds. Transformed bacterial cells were shaken at 175 rpm and 37°C for 1 h. X-Gal (40 µL of 40 mg/mL) was spread in LB agar plates containing 50 µg/mL kanamycin, and then bacterial cells were inoculated. The bacterial cells were grown overnight at 37°C, and single white colonies were picked up with a toothpick prior to transferring to 10 mL of Lysogeny broth (LB) liquid culture containing 50 µg/mL kanamycin. Afterwards, the bacterial cells were shaken overnight at 175 rpm at 37°C. For plasmid isolation, a PureLink® Quick Plasmid Miniprep Kit (Life Technologies, CA, USA) was used. Isolated plasmids were subjected to restriction digestion in order to confirm the cloning of the P5βR gene. The restriction digestion reaction contained 5 µL of autoclaved bidistilled water, 2 µL of DNA plasmid, 2 µL of EcoRI buffer and 1 µL of EcoRI enzyme for 10-µL reaction volumes for each plasmid. The restriction digestion reaction was carried out at 37°C for 1 h. The reaction was terminated by adding 2 µL of 6×loading dye and electrophoresed as mentioned above. Positive clones were sequenced for sequence confirmation.

FIGURE 5. *Digitalis* species from Anatolia: *D. ferruginea* subsp. *ferruginea*. A. Habit, B-E. Inflorescence (variations in flower colour), F. Fruits (photographed by Eker).

Selection of Bacteria and Plasmid Isolation

After incubation overnight, white (positive) colonies that had formed on the petri dishes were selected carefully. A single white single colony was transferred to aculture tube containing 3 mL of LB medium (10 tubes in total for each species). After another overnight incubation of selected colonies at 37°C in an orbital shaker, plasmid DNA was isolated using a PeqGOLD Plasmid Miniprep Kit (Peqlab GmbH, Erlangen, Germany), following the manufacturer's

instructions. In order to determine which colony should be sequenced, digestion of plasmid was carried out using 8 µL of plasmid DNA, 1 µL of enzyme *Eco*RI and 1 µL of reaction buffer (Fermentas GmbH, St. Leon-Rot, Germany). Components in a PCR tube were mixed briefly and incubated at 37°C for 1 h in a thermocycler (Eppendorf, Hamburg, Germany). After subsequent gel electrophoresis of the samples, positive clones were sequenced by Eurofins MWG/ Operon AG (Stuttgart, Germany).

FIGURE 6. Digitalis species from Anatolia: D. ferruginea subsp.schischkinii. A. Habit, B-E. Inflorescence (variations in flower colour), F. Fruits (photographed by Eker).

In Silico Analyses

The data were analysed using the ApE-A plasmid Editor v1.17 and GenBank™ database (http://blast.ncbi.nlm.nih.gov.BLAST.cgi) for BLAST search. Phylogenetic trees of *Digitalis* species were constructed using the maximum parsimony (MP) and maximum likelihood (ML) algorithms provided by the MEGA 5.5 software package (Tamura *et al.* 2011, Hall 2011). MP was employed with a test of phylogeny with 1000 bootstrap (BS) values. The MP search method selected was Close-Neighbour-Interchange (CNI) with the default setting of 10 trees.

ML was employed with the following settings: test of phylogeny was the BS method with 1000 replications. The substitution model was based on Kimura's 2-parameter model; rates among sites were shown using a gamma

distribution model with five discrete gamma categories; tree inference options ML were set to Nearest-Neighbour-Interchange (NNI), the branch swap filter was set to the default. In all heuristic searches, 24 sequences (including an outgroup) were used.

Results

Branching Pattern of sect. *Globiflorae*

For the evolutionary history of *Digitalis* species in Anatolia, nucleotide sequences of functional homologues of P5βR proteins were deduced for *D. cariensis*, *D. trojana*, *D. lamarckii*, *D. lanata* subsp. *lanata*, *D. ferruginea* subsp. *ferruginea* and *D. ferruginea* subsp. *schiscKhinii*. Phylogenetic trees were constructed using nucleotide sequences of the cDNAs of P5βRs from *Digitalis* species. In order to infer the evolutionary history of the aforesaid species, two different algorithms based on MP and ML were used to construct phylogenetic trees using 29 specimens including one outgroup, *Erysimum rhaeticum* (Schleicher ex Hornemann 1815: 613) Candolle (1821: 503), from Brassicaceae. Of those data sets, six taxa collected from their natural habitats in Turkey are new in this study, and have not been investigated in detail in previous publications (Bräuchler *et al.* 2004, Herl *et al.* 2008). Although Herl *et al.* (2008) constructed a phylogenetic tree based on MP using sequences of *D. cariensis* and *D. lanata*, these two sequences do not reflect their original geographical distributions (Fig. 7).

The reading frame of the *P5βRs* gene contains about 1,170 nucleotides equal to 389 amino acids. Of those nucleotides, 1043 sites (89.1%) were conserved, 110 sites (10.9%) were variable, including 52 parsimony informative characters, and 75 were singleton sites within all *Digitalis* data set. The G + C content was about 47.9%. Phylogenetic analysis was inferred using the MP method. Tree number 9 is selected among the 11 MP trees (length = 513) as seen in Fig. 8. The consistency index is 0.64, the retention index is 0.78, and the composite index is 0.71 for all sites, and parsimony-informative sites are 0.50. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is also shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 (default set) in which the initial trees were obtained by the random addition of sequences (1000 replicates). Similarly the ML method based on Kimura's 2-parameter model was investigated. The tree with the highest log likelihood (-4044.08) is shown in Fig.8. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.7413)].

In the present study, *D. cariensis* and *D. lamarckii* are found to be very closely related species and isolated clearly from other sister taxa (BS 100% in MP; 80% in ML tree) under sect. *Globiflorae* in which *D. trojana* and *D. lanata* subsp. *lanata* collected from Gelibolu were closely positioned (Fig. 8). However, the phylogenetic position of *D. lanata* subsp. *lanata* originating from Bulgaria might make it a separate taxon and weakly supported in this clade. Based on the MP tree, *D. ferruginea* taxa distributed in the Balkan Peninsula and another node branching through several polytomic groups (50%<BS) distributed in North Anatolia were clearly separated from each other (BS<74%, Fig. 8).

Morphological and Geographical Analyses of Taxa

With respect to the morphological comparison of the taxa described in sect. *Globiflorae* (Table 3; Figs. 1–6), *D. ferruginea* subsp. *ferruginea* and *D. ferruginea* subsp. *schischkinii* are clearly different from the other taxa in sect. *Globiflorae* by having glabrous sepals with scarious margins (and rarely ciliate margins). Despite the high morphological similarity between the two taxa, there are some minor differences in respect to the geographical distribution, plant size and flower colours. *Digitalis ferruginea* subsp. *ferruginea* has relatively large flowers (15–23 mm) with a wide middle lobe of the lower lip (5–8 mm) in contrast to the relatively small flowers (10–16(–20) mm) with a narrow middle lobe of the lower lip (3–5 mm) in *D. ferruginea* subsp. *schischkinii*. Although both species show wide variation in corolla colour on the outer surface, there is consistency in colour patterns. For example, reddish-brown, rusty to yellowish-brown in *D. ferruginea* subsp. *ferruginea* and yellow, ginger yellow to greenish-yellow, with or without tinged red colour in *D. ferruginea* subsp. *schischkinii* are such dominant and typical characters. Geographically, *D. ferruginea* subsp. *ferruginea* is distributed in Thracia and outer Anatolia including the Aegean, Mediterranean and western Black Sea regions in Turkey. The eastern corners of the Küre and Ilgaz mountains in the north act as a barrier between the two taxa. Thus, *D. ferruginea* subsp. *ferruginea* is replaced by another subspecies, *D. ferruginea* subsp. *schischkinii*, towards northeastern Anatolia and the West Caucasus. In the south, *D. ferruginea* subsp. *ferruginea*is restricted to the western Taurus and Amanos mountains. In this region, the expansion of the plant continues towards to the south, including Syria and Lebanon. Both subspecies may be found from sea level up to 2700 m on roadsides, in forest clearings, and on bushy and grassy slopes in Turkey.

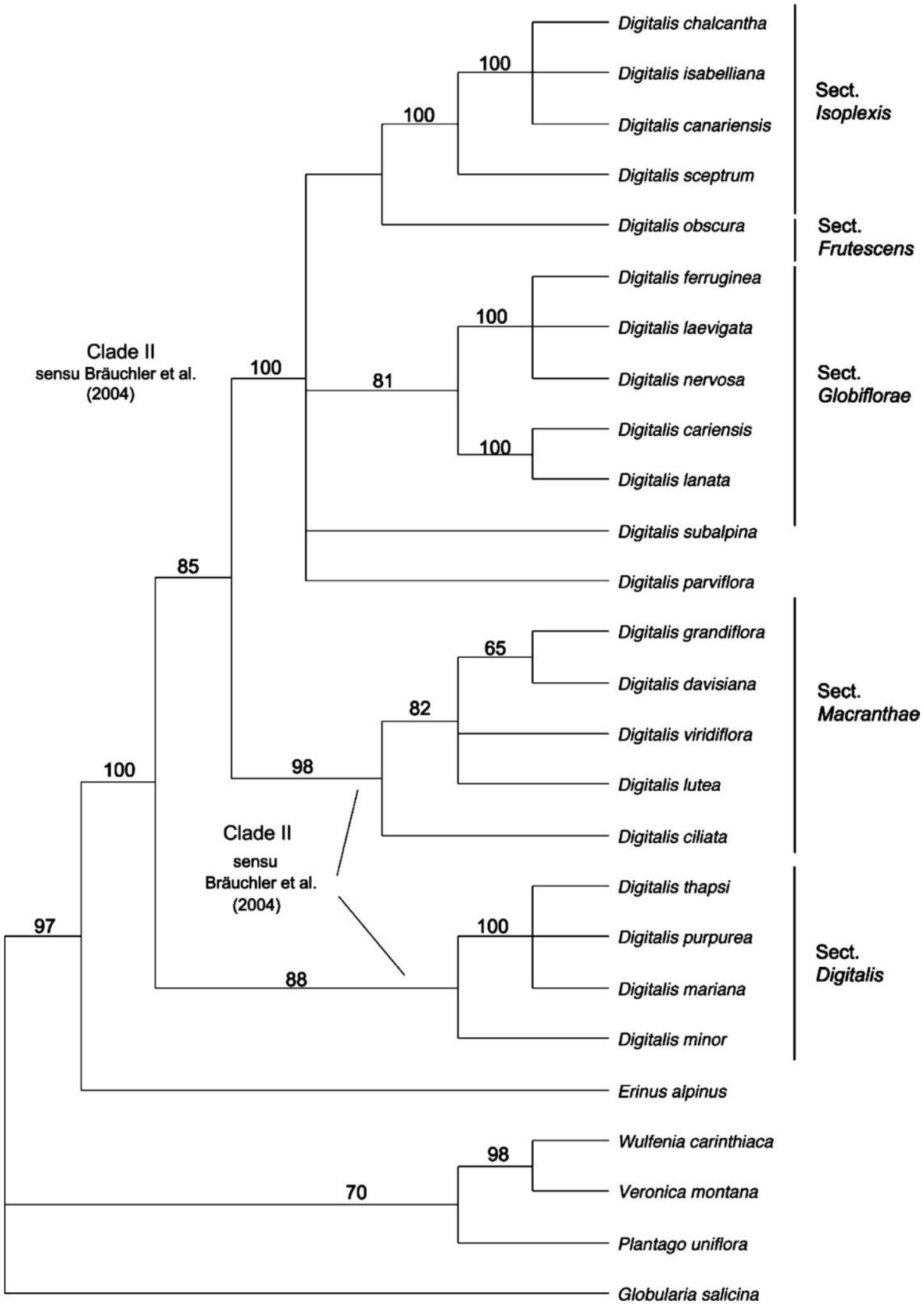

FIGURE 7. Consensus tree of Herl *et al.* (2008).

TABLE 3. Morphological descriptions of the taxa in sect. *Globiflorae* according to Werner (1960), Davis (1978) and present study

Taxa	Descriptions		
	Werner, 1960	Davis, 1978	Present study
D. cariensis	Median leaves 6–14 mm broad; Raceme dense; Corolla 11–15(–17) mm long; Lower corolla lobe 5–8 mm long.	Median leaves 7–10 mm broad, linear, glabrous; Raceme dense; Corolla usually 10–15 mm long; Lower corolla lobe whitish, usually 4–7 mm, ovate to ovate-oblong, not narrowed below; Capsule 9–10 mm including beak; 800–1700 m asl.	Median leaves 9–18 mm broad, lanceolate; Basal leaves linear and gradually broading to blunt apex, 15–35 cm × 4–13 mm; Inflorescence axis pubescent only above, glabrous below; Raceme dense; Corolla 8–15 mm long; Lower corolla lobe yellowish-white with distinct reddish-brown veins, 3–7 mm; Capsule 10–15 mm including beak; 800–1700 m asl.
D. lamarckii	Median leaves 4–8(–11) mm broad; Raceme very lax; Corolla 20–27(–34) mm long; Lower corolla lobe 10–14(–16) mm long.	Median leaves 4-10 mm broad, linear, glabrous; Raceme usually lax; Corolla usually (20–)25–30(–35) mm long; Lower corolla lobe whitish, 10–14(–16) mm, oblong-obovate, narrowed below; Capsule 10–14(–16) mm including beak; 0–1500 m asl.	Median leaves 8-14 mm broad, lanceolate; Basal leaves obovate to linear, 5–20(–25) cm × 4–13 mm; Inflorescence axis pubescent only above, glabrous below; Raceme lax; Corolla 15-30 mm long; Lower corolla lobe white with slightly reddish-brown or yellowish-brown veins, 7–12 mm; Capsule 14–20 mm including beak; 600–1500 m asl.
D. lanata* subsp. *trojana	Median leaves 6–20 mm broad; Raceme rather dense; Corolla (14–)16–25 mm long; Lower corolla lobe, (4–)5–9 mm long.	Median leaves 10–15(–20) mm broad, linear-lanceolate, glabrous or rarely sparsely ciliate; Raceme moderately dense; Corolla usually (16–)20–25 mm long; Lower corolla lobe whitish with reddish-brown veins, 7–13 mm, oblong, obtuse, not or scarcely narrowed below; Capsule 13–15 mm including beak; 90–800 m asl.	Median leaves 7–13 mm broad, oblong-lanceolate or oblanceolate; Basal leaves obovate, 5–20 cm × 4–18 mm; Inflorescence axis and bracts ciliate to slighty villouse; Stem generally green, sometimes slightly reddish below; Raceme dense; Corolla 15–25 mm long; Lower corolla lobe white with slightly mustard-coloured veins, 7–13 mm; Capsule 14–20 mm including beak; 200–800 m asl.
D.lanata* subsp. *lanata	Median leaves (9–)11–30(–45) mm broad; Raceme very dense to lax; Corolla (17–)20–25(–30) mm long; Lower corolla lobe (9–)10–13(–14) mm long.	Median leaves 10–20 mm broad, oblong-lanceolate or oblanceolate, glabrous or with margin softly ciliate; Raceme very dense; Corolla usually 18–25 mm long; Lower corolla lip lingulate, 10–13 mm, oblong to ovate; 50–100 (or more) m asl.	Median leaves 9–16 mm broad, lanceolate; Basal leaves obovate, 5–20 cm × 7–15 mm; Inflorescence axis and bracts densely villouse; Stem often purplish; Raceme dense; Corolla 17–25 mm long; Lower corolla lobe white with slightly mustard-coloured veins, 8–13 mm; Capsule 15–20 mm including beak; 0–100 m asl.
D. ferruginea* subsp. *ferruginea	Leaf shape and leaf indumentum, inflorescence and flower colour (light yellow to dark rusty) highly variable	Cauline leaves oblong to oblong lanceolate or linear, often pubescent on margin and on veins of lower surface; Raceme long, many flowered, fairly to very dense, axis glabrous; Corolla 18–34 mm; middle lobe of lower lip c. 8 mm broad; Capsule glabrous; 0–2700 m asl.	Corolla relatively bigger (15–23 mm) with wide middle lobe of lower lip (5–8 mm). Corolla colour on outer surface reddish-brown, rusty to yellowish-brown. It is distributed in Thracia and the outer Anatolia including Aegean, Mediterranean and West Black Sea regions in Turkey. Capsule glabrous; 0–2700 m asl.
D. ferruginea* subsp. *schiscкhinii	The main difference from type subspecies is smaller flowers	Cauline leaves oblong to oblong lanceolate or linear, often pubescent on margin and on veins of lower surface; Raceme long, many flowered, fairly to very dense, axis glabrous; Corolla (8–)12–18 mm; middle lobe of lower lip c. 4 mm broad; Capsule glabrous; 1200–2000 m asl.	Corolla relatively smaller (10–16(–20) mm with narrow middle lobe of lower lip (3–5 mm). Corolla colour on outer surface yellow, ginger yellow to greenish-yellow with or without tinged red colour. It spreads towards North-eastern Anatolia and West Caucasus. Capsule glabrous; 100–2200 m asl.

Digitalis cariensis has the smallest corolla (8–15 mm) and lower corolla lobe (3–7 mm) among the close taxa. The other remaining taxa, including *D. lamarckii*, *D. lanata* subsp. *lanata* and *D. trojana*, possess a large corolla (15–30 mm) and lower-corolla lobe (7–13 mm). The colour of the lower-corolla lobe in *D. cariensis* is yellowish along with distinct reddish-brown veins, whereas the other aforesaid taxa's lower-corolla lobe is white or creamy coloured with slightly reddish- or yellowish-brown veins. The basal leaves of *D. cariensis* are linear (15–35 cm), extending to the blunt apex, while *D. lanata* subsp. *lanata* and *D. trojana* have obovate leaf types (5–20 cm in length). Basal leaves of *D. lamarckii* are obovate to linear, 5–20 (–25) cm in length. Moreover, *D. cariensis* has a smaller capsule (10–15 mm) compared to all other remaining taxa (14–30 mm). *Digitalis lamarckii* has a lax stem in contrast to the dense raceme of the remaining taxa. *Digitalis lanata* subsp. *lanata* and *D. trojana* have often densely villous to ciliate inflorescence, while it is sparsely ciliate in *D. cariensis* and *D. lamarckii.* As for habitat preference, all taxa prefer the edge of forest clearings and roadsides. However, *D. cariensis* and *D. lamarckii* mostly showa natural distribution at altitudes above 750 m, while *D. lanata* and *D. trojana* are distributed at elevations below 750 m. The distribution patterns of *D. cariensis* and *D. lamarckii* at low altitudes are rarely seen as a continuation of the plant's main distribution in mountain ranges. *Digitalis cariensis* is found on limestone and serpentine slopes of clearings and roadsides in coniferous and *Quercus* Linnaeus (1753: 994) forests. Similarly, *D. lamarckii* spreads on rocky or shaly slopes of openings in *Pinus* Linnaeus (1753: 1000) and *Quercus* forests. *Digitalis cariensis* and *D. lamarckii* are solitary species with a random distribution within their habitats. *Digitalis lanata* is mostly adapted to bushy hillsides in macchie, scrub and phrygana up to 100 m rather than to rocky and shaly slopes. However, *D. lanata* spreads to higher altitudes in the Ganos and Istranca mountains up to 550 m on roadsides. The habitat preferences of *D. trojana* are moderately shally slopes and roadsides in coniferous and *Quercus* forests between 200 and 750 m on the slopes of Mount Ida (Kazdağı).

Taxonomic Treatment

Digitalis lanata Ehrh. subsp. ***trojana*** (Ivanina) Yücesan & Eker, ***stat. nov.***

Basionym: *Digitalis trojana* Ivanina (1955: 263).

Homotypic synonym: *Digitalis cariensis* Boiss. ex Benth. subsp. *trojana* (Ivanina) Werner (1960: 244).

Type: [Turkey B1 Balıkesir] Troja, Mt. Ida: in monte Kapu-Dagh, 12 June 1883, *Sintenis 461* (holotype: LE; isotypes: K, E00326081!, S10-25691!).

Diagnostic Key of the Taxa in sect. *Globiflorae* in Turkey

1. Sepals glabrous; with a conspicuous scarious border..... *D. ferruginea* (2)
- Sepals hairy; without a scarious border..... (3)
2. Corolla 15–23 mm; middle lobe of lower lip 5–8 mm broad; outer surface of corolla reddish-brown, rusty to yellowish-brown; distributed in outer Anatolia except northeast parts to Balkans ..... *D. ferruginea* subsp. *ferruginea*
- Corolla 10–16(−20) mm; middle lobe of lower lip 3–5 mm broad; outer surface of corolla yellow, ginger yellow to greenish-yellow with or without red tinge; distributed in northeast Anatolia to Caucasus..... *D. ferruginea* subsp. *schischkinii*
3. Corolla less than 15 mm long; lower corolla lobe less than 7 mm..... *D. cariensis*
- Corolla more than 15 mm long; lower corolla lobe more than 7 mm ..... (4)
4. Raceme lax; inflorescence axis ciliate only above..... *D. lamarckii*
- Raceme dense; inflorescence axis densely ciliate and/or villous ..... *D. lanata* (5)
5. Inflorescence axis densely villous; stem often distinctly purplish below ..... *D. lanata* subsp. *lanata*
- Inflorescence axis ciliate to villous; stem generally green, sometimes slightly reddish below ..... *D. lanata* subsp. *trojana*

Discussion

Phylogeny of sect. *Globiflorae*

Based on the phylogenetic trees created from the data set, it is noteworthy that *D. cariensis* and *D. lamarckii* appear as a sister group, clearly separated from a node rooting another closely related group in which *D. trojana* and *D. lanata* subsp. *lanata* are located. Werner's *cariensis* alliance, including *D. cariensis* subsp. *cariensis*, *D. cariensis* subsp. *lamarckii* (Ivanina 1955: 260) Werner (1960: 244) and *D. cariensis* subsp. *trojana* (Ivanina 1955: 263) Werner (1960: 244), is not consistent with our findings. Based on our topology and network estimations (data not provided), the following implications regarding *Digitalis* evolution might be considered for sect. *Globiflora*e: (1) *D. cariensis*

and *D. lamarckii* might have been separated from an ancestor that rooted *D. trojana*; (2) *D. lanata* subsp. *lanata* collected from Gelibolu and *D. trojana* from Mount Ida were strictly bifurcated from the node rooted to *D. cariensis* and *D. lamarckii*; *D. lanata* subsp. *lanata* might be evaluated as the most ancestral species; (3) *D. trojana* might also be evaluated as a new combination under *D. lanata*, *i.e.*, *D. lanata* subsp. *trojana*. Similarly, Bräuchler *et al.* (2004) reported combined sequence data sets of *ITS* and *trnL-F* (BS 100%) in their phylogenetic analysis. Although they showed a close relationships between *D. lanata* (*D. lanata* subsp. *lanata* and *D. lanata* subsp. *leucophaea*) and *D. trojana*, they did not mention the phylogenetic position of *D. cariensis* or *D. lamarckii* at all. For *D. trojana*, both Bräuchler *et al.* (2004) and Herl *et al.* (2008) followed Werner's *cariensis* alliance, and evaluated *D. trojana* as *D. cariensis* Boissier ex Bentham (1846: 450) subsp. *trojana* Werner (1960: 244).

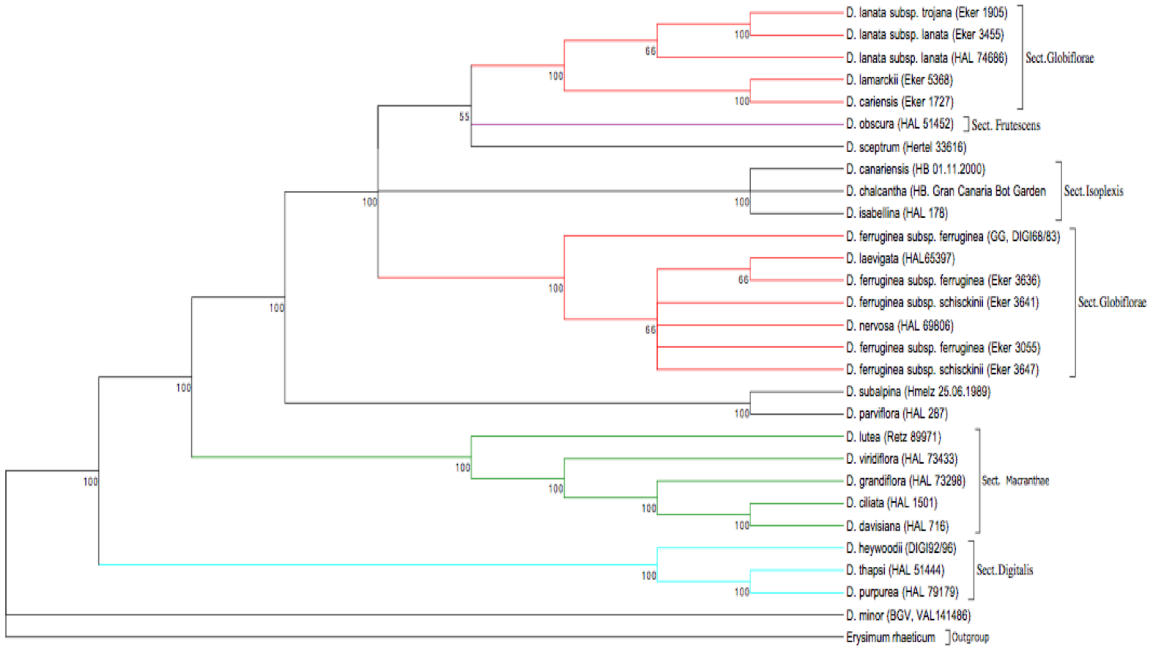

FIGURE 8. Bootstrap consensus tree based maximum parsimony analysis (up) and maximum likelihood method of *Digitalis* taxa (down). Phylogenetic trees were constructed using MEGA 6.06. Voucher numbers of specimens represented in parenthesis.

Based on our literature survey, there is only one study in agreement with Werner's *cariensis* alliance in which a chemotaxonomic approach was mentioned. In this respect, Wichtl & Huesmann (1982) treated *D. trojana*, *D. cariensis* and *D. lamarckii* as three subspecies under *D. cariensis*, *i.e*. subsp. *trojana*, subsp. *lamarckii* and subsp. *cariensis*, rather than as independent species, since they had similar cardenolide patterns quantitatively in their ground leaves. Even though this study provides an approach for recognising close species under the same section based on the cardenolide profiles as a fingerprint pattern, more studies are required to reach a consensus on the taxonomical rankings of species. Moreover, cardiac glycosides are produced in a limited number of genera in many unrelated families (i.e. Plantaginaceae, Scrophulariaceae, Convallariaceae, Apocynaceae, Ranunculaceae, Celastraceae, Brassicaceae, Hyacinthaceae, Fabaceae, Moraceae and Tiliacae), and even in some animals such as toads and beetles (Kreis & Müller-Uri 2010, Agrawal *et al.* 2012). Wink *et al.* (2010) reported that these compounds cannot be recommended as phylogenetic/chemotaxonomic markers at the family level as seen in iridoid glycosides, products of the isoprenoid biosynthetic pathway. Since cardenolides appear to have evolved independently on a number of occasions, they might be evaluated as a marker, but partially under section or subsection level of the genus containing cardenolides. Thus, cardenolides might be a useful tool for the representation of total contents underlying medicinally important A-, B- and/or C-types.

Bräuchler *et al.* (2004) and Herl *et al.* (2008) reported that sect. *Globiflorae* contained several taxa that have a main centre of diversity in Anatolia. The phylogenetic analysis of *D. ferruginea* by Bräuchler *et al.* (2004) had also indicated polyphyletic speciation in the western Caucasus; furthermore, they stated it was appropriate to raise *D. ferruginea* subsp. *schischkinii* to the rank of species, *D. schischkinii* Ivanina (1946: 204). However, Herl *et al.* (2008) did not investigate *D. ferruginea* taxa distributed in North Anatolia. Instead, they reconstructed a strict consensus tree in which Balkan origin *D. ferruginea* subsp. *ferruginea* was grouped together with *D. laevigata* and *D. nervosa*. In our study, with the inclusion of a new data set from North Anatolia for *D. ferruginea* taxa, *D. ferruginea* distributed in the Balkan Peninsula might be evaluated as the most recent ancestor within this large clade. Nevertheless, new samples from Mid-Europe through the Balkan Peninsula might clarify the branching pattern of *D. ferruginea* taxa.

Morphological and Biogeographical Implications

Morphological analysis is another auxiliary method for determining the formation of sect. *Globiflorae*. Werner (1960) decided on a nomenclature grouping of Anatolian endemics (*D. cariensis*, *D. lamarckii* and *D. trojana*) into a single taxon, in contrast to the separate species level of Ivanina's system (1955). Additionally, Davis (1978) distinguished narrower and linear median leaves in *D. cariensis* and *D. lamarckii*, and lanceolate median leaves in *D. trojana* and *D. lanata*. Moreover, he specified the ciliate bracts in *D. lanata* only, not in the others. When compared with our morphological observations, these character sets in sect. *Globiflorae* do not seem discriminative in order to extrapolate a clear taxonomical rank. Instead of hairiness of median leaves and bracts, the following characters including shape and length of basal leaves should be used for the classification of taxa. Based on the morphological observations shown in Table 3, in addition to inflorescence axis pubescence, flower density of inflorescence, and size of flower parts as described by Werner (1960) and Davis (1978), we also recommend the use of general texture of basal leaves (*i.e*. size and shape) as a discriminative character for the species key with respective descriptions. It is noteworthy that *D. cariensis* possesses the smallest corolla, lower-corolla lobe, and capsule. On the other hand, it has longer linear basal leaves than the other close taxa (*D. trojana*, *D. lamarckii* and *D. lanata*). Thus, *D. cariensis* can be evaluated as a distinct species. *Digitalis lamarckii*, having lax racemes and less inflorescence pubescence, is easily distinguished from *D. lanata* and *D. trojana* with dense racemes and inflorescence pubescence.

In the present study, a high morphological similarity between *D. lanata* subsp. *lanata* and *D. trojana* were observed. However, the only significant difference is found in the density and type of the hair structures. The highest density of villous hair structure is observed in *D. lanata* subsp. *lanata*. This trichome structure might be an adaptive character against drought by reducing absorbance of solar radiation, and increasing the leaf surface boundary layer to facilitate condensation of air moisture onto the plant surface. The density of villous hairs gradually decreases in *D. trojana*, whereas the upper inflorescence axes of both *D. lamarckii* and *D. cariensis* are only pubescent (ciliate, non-villous), and the lower inflorescence axes are almost glabrous as well. Phylogenetic investigations reveal that *D. lanata* might be the oldest or most ancestral species in sect. *Globiflorae*. As to the position of *D. trojana* as a refuge on Kaz Dağı (Mount Ida), it might be placed under *D. lanata* (*i.e*. *D. lanata* subsp. *trojana*). Davis (1978) pointed out that Werner's records of *D. lamarckii* from Southwest Anatolia could be considered as *D. cariensis*. This concept is also consistent with our observations made on several field trips. The findings in Southwest Anatolia indicated Werners' misapprehension in his taxonomy. The different varieties of *D. cariensis* in Southwest Anatolia could be possibly misinterpreted as *D. lamarckii*. Otherwise, as Davis (1978) stated, the combination of all three taxa under

D. lanata would be a more plausible outcome rather than Werner's *D. cariensis* concept (Werner 1960, 1964, 1965). In the present study, the Balkan Peninsula can be regarded as an important gateway to Anatolia for the speciation of sect. *Globiflorae* (Fig. 9). This hypothesis also bears some similarities with that proposed by Bräuchler *et al.* (2004) indicating that glacial influence forced *D. purpurea* and its closely related subspecies to adapt mainly to the Iberian Peninsula. During the glacial period, Thrace and Northeastern Anatolia served as an entrance to the rest of Anatolia for boreal flora (Eken & Ataol, 2006). Similarly, glaciations during the ice age period could presumably have influenced the genus *Digitalis* to move from the Balkan Peninsula towards Anatolia (Fig. 9). The complexity in classification can only be minimized when we find an accurate tree that most closely approximates what might have possibly happened in the past. To achieve this, vegetation patterns of the species should be well understood. For example, *D. lanata* subsp. *lanata* and *D. lanata* subsp. *trojana* might be regarded as good examples for allopatric speciation, when considering local endemism in Edremit Gulf wherein *D. lanata* subsp. *trojana* is distributed mainly on Mount Ida up to 750 m, and the island of Thassos for natural habitats of *D. lanata* subsp. *leucophaea*. For *D. cariensis* and *D. lamarckii*, when their habitats are taken into account, they grow naturally in areas where open *Pinus* and *Quercus* forests and rock or pebble slopes with varying attitudes between 750 and 1800 m are mainly present. Otherwise, *D. lanata* grows on hillsides in macchie or shrub between 0 and 100 m and *D. trojana* spreads between 200 and 750 m altitudes on Mount Ida, Turkey. Due to the adverse effects of the water level at the coast during the Pleistocene, *D. cariensis* and *D. lamarckii* might have adapted to high altitudes too dry to support a forest for survival. This hypothesis is correlated with the glaciations period in Anatolia (~120,000 years ago; Lambeck 1995). During the early period of glaciations, we suppose that Anatolia was an important refuge area for the entrance of many *Digitalis* species belonging to sect. *Globiflorae* from the Balkan Peninsula. The members of *Globiflorae*, including *D. ferruginea* taxa, might have evolved due to the presence of the following three mountainous terrains: West Black Sea Mountains in North Anatolia, the West Anatolian Mountains, and finally the Taurus Mountains in South Anatolia (Sengör & Yilmaz 1981; Demirsoy 1996). We can also attribute the high diversification and endemism rate in sect. *Globiflorae* to those mentioned fragmentations in Anatolia. Distribution of *D. ferruginea* taxa throughout the Balkans and Anatolia might also have resulted in new speciation through Southern Europe in the form of *D. laevigata* and through the north of Iran and the Caucasus in the form of *D. nervosa*.

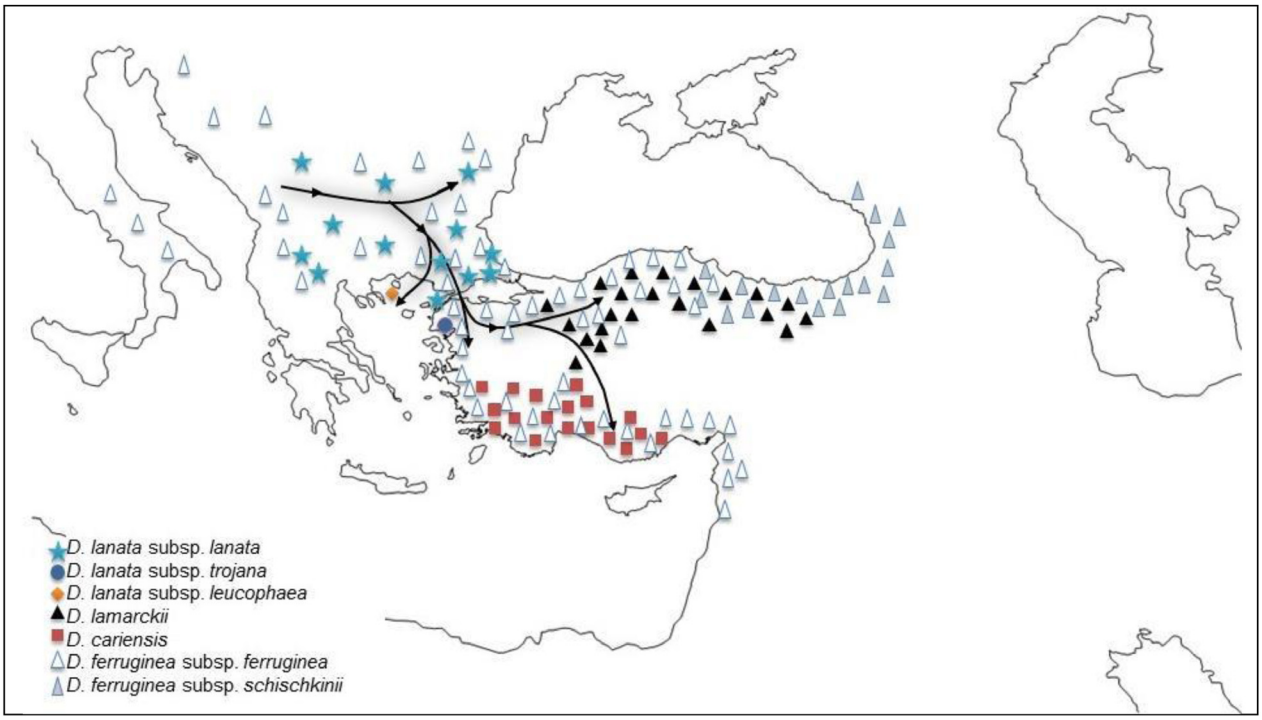

FIGURE 9. An hypothetical map for branching pattern of sect. *Globiflorae.* Arrows represent direction of the entrance of ancestral clade(s) through Anatolia.

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