Contribution to chromosome numbers and phylogeny of Turkish Vincetoxicum Wolf (Apocynaceae, Asclepiadoideae)

Seher GÜVEN 1,*, Serdar MAKBUL 2 & Kamil COŞKUNÇELEBİ 3

1,2 Recep Tayyip Erdogan University, Faculty of Sciences and Arts, Department of Biology, 53100, Rize, Turkey.
3 Karadeniz Technical University, Science Faculty, Department of Biology, 61080 Trabzon, Turkey.

* Corresponding author: cakmakseher@hotmail.com
2 Email: smakbul@hotmail.com
3 Email: kamil@ktu.edu.tr

Abstract. We report chromosome counts for ten taxa of Vincetoxicum sensu stricto (s. str.) (Apocynaceae) from Turkey (of which two are endemic), including the first chromosome counts for V. canescens subsp. pedunculata, V. funebre, V. fuscatum subsp. boissieri, V. parviflorum and V. tmoleum. Two taxa of V. fuscatum proved to be tetraploid (2n=44) and the remaining eight taxa diploid (2n=22). Molecular phylogenetic analyses based on nrDNA (ITS) and cpDNA (trnT-trnL) (including 31 newly generated sequences) confirm the position of the Turkish Vincetoxicum in the Vincetoxicum s. str. clade. Vincetoxicum fuscatum, V. parviflorum, V. speciosum, as well as the Turkish endemic V. fuscatum subsp. boissieri, were clearly resolved as species-level clades, whereas the delimitation of the rest of the Turkish taxa was less clear based on molecular data.

Keywords. Cytology, molecular phylogeny, Turkey, Vincetoxicum.

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Introduction

Vincetoxicum Wolf is one of the largest and most widespread genera of the subfamily Asclepiadoideae (Apocynaceae). Molecular data indicated that Vincetoxicum sensu lato (s. lat.), comprising ca 150 species, is widely distributed from Australasia and the Far East to Africa, Europe, and North America (Liede-Schumann et al. 2016). The well-defined Vincetoxicum s. str. clade with ca 45 species extends from the Far East via the Asian mountain ranges into Central Europe and was also introduced in North America (Liede-Schumann et al. 2016). Vincetoxicum displays the flowers typical of the subfamily with gynostegium and five pollinaria, its sterile corona is staminal and characteristically composed of five fleshy lobes separated near the base or partly connected by smaller interstaminal parts (Liede 1996). In addition, most Vincetoxicum individuals have been determined to be poisonous to humans, and some
have been used in conventional and folk medicine as well as in modern medicine (Zaidi & Crow 2005; Mansoor et al. 2011).

*Vincetoxicum* was considered to be a systematically difficult genus due to the complex floral features which could be variable even at the species level (Browicz 1978). The genus contains many morphologically close taxa, and there have long been various interpretations over the taxonomic distinction of *Vincetoxicum* from its closely related genera in the same tribe (Asclepiadeae); *Cynanchum* L. (subtribe Cynanchinae K.Schum.) and *Tylophora* R.Br. (subtribe Tylophorinae), which are known by their similar corona, gynostegium and pollinarium structures (Liede-Schumann et al. 2012). *Vincetoxicum* was recognized as a separate genus from *Cynanchum* by some taxonomists (Pobedimova 1952; Grossheim 1967; Rechinger 1970; Markgraf 1972; Browicz 1978; Ali 1983), while others preferred to classify it under *Cynanchum* at the rank of subgenus (Domin 1928) or section (Tsiang & Li 1977; Forster 1991). Some morphological (Liede 1996; DiTommaso et al. 2005; Yamashiro et al. 2008), palynological (Chang et al. 2012; Feng et al. 2012; Shah & Ahmad 2014; Yaseen & Perveen 2014), chemical (Stærk et al. 2002; Zaidi & Crow 2005, 2012; Mansoor et al. 2011), cytological (Liede-Schumann et al. 2012) and molecular (Sennblad 1997; Civeyrel et al. 1998; Liede 2001; Liede et al. 2002; Yamashiro et al. 2004; Liede-Schumann et al. 2012, 2016) studies, including a few taxa of *Vincetoxicum*, have been carried out on Asclepiadoideae in order to contribute to the taxonomy of the subfamily. Recently, Liede-Schumann et al. (2012, 2016), based on molecular data (nrDNA and cpDNA markers), indicated that in the subtribe Tylophorinae, containing *Biondia* Schltr., *Blyttia* Arn., *Diplostigma* K.Schum., *Goydera* Liede, *Pentatropis* R.Br., *Pleurostelma* Baill., *Rhyncharrhena* F.Muell., *Tylophora* and *Vincetoxicum*, all genera, except *Pentatropis*, are non-monophyletic. Therefore, the researchers suggested to merge the remaining eight genera of the subtribe Tylophorinae into a single genus, *Vincetoxicum* s. lat. (Liede-Schumann et al. 2012, 2016). Consequently, Liede-Schumann & Meve (2018) formally transferred the members of these genera to *Vincetoxicum* s. lat., the oldest established genus name.

Browicz (1978), who recognised six species and four subspecies, excluding doubtful records, presented a comprehensive treatment of *Vincetoxicum* s. str. in Turkey. Since then, Güner (2012) listed ten taxa of *Vincetoxicum* s. str. from Turkey, including endemics (*V. fuscatum* Rchb. subsp. *boissieri* (Kusn.) Browicz and *V. parviflorum* Decne.). *Vincetoxicum* s. str. grows in various habitats ranging from dry river valleys, open rocky slopes, steppes and mountain slopes, to shrubland and *Quercus*-dominated forests in Turkey (Browicz 1978). Until now, a few anatomical and seed micro-morphological (İlçim et al. 2010), ethnobotanical (Doğan 2008; Altundağ & Öztürk 2011), pharmaceutical (Özay 2013), and palynological (Güven et al. 2015) studies have been reported on Turkish *Vincetoxicum* s. str. taxa.

Chromosome number data remain valuable today in systematics, and establishing which plants are polyploids and which are diploids is important to allow for a better understanding of plants to be included in phylogenetic studies. Polyploidy, widely accepted as a mechanism of reproductive isolation and plant speciation, has been an important force shaping the evolutionary history of vascular plants. It is estimated that between 15% and 30% of speciation in angiosperms results from polyploidy. Over the last decade, there has been an increase in studies investigating how polyploids influence phylogenetic community structure by combining ploidal information with phylogenetic analyses of plant communities. The polyploid and diploid species have been identified as having phenotypic differences and molecular variations associated with whole genome duplication. While these differences are considered to increase the ecological success of polyploids in novel communities, it remains relatively unexplored whether polyploids are indeed better competitors in a community context (Gregg et al. 2017; Gaynor et al. 2018).

For nearly a century, botanists in particular, have been interested in the determination and documentation of chromosome numbers which have been extensively utilized as an important phylogenetic character in the context of cytotaxonomy (Rice et al. 2015). Although these data have been documented along the years, to date, chromosome number data of only approximately 25% of flowering plants have been
determined (Stace 2000; Garbari et al. 2012). Despite the great floristic richness of Turkey, only 15% of the vascular plant taxa have had their chromosome number investigated (Vladimirov et al. 2015).

The aim of the present study was: 1) to report the chromosome number of Turkish representatives of *Vincetoxicum* s. str. since very few counts have been reported in the literature previously; and 2) to contribute to the phylogenetic position of *Vincetoxicum* s. str. (of which two are endemic) based on newly generated sequences from Turkish accessions.

**Material and methods**

**Sampling**

Locality information of samples used for chromosome counting and molecular studies is presented in Appendix 1. Of these, 16 representatives belonging to 10 Turkish taxa of *Vincetoxicum* s. str. were used in somatic chromosomal studies. In total, 59 accessions (*Vincetoxicum* s. str. (53), *Cionura erecta* L. (3), *Cynanchum acutum* L. (2) and *Gomphocarpus fruticosus* (L.) W.T.Aiton (1)) were used for molecular analysis (Appendix 1). Of these, one *Cynanchum*, three *Cionura* and 31 *Vincetoxicum* s. str. members were collected by the present authors from their natural habitats in Turkey (Appendix 1). All specimens were first processed using the standard herbarium techniques given by Woodland (1997); they were identified using the Flora of Turkey (Browicz 1978), Flora of Russia (Pobedimova 1952), Flora Europaea (Markgraf 1972), and stored at the Herbarium of Recep Tayyip Erdogan University, Department of Biology (RUB).

**Cytological analyses**

For somatic chromosomal examination, the mature seeds were germinated on wet filter paper in Petri dishes at +27°C. Active roots were cut at 1–1.5 cm from the tips and these were pretreated for 16 hours in α-monobromonaphthalene at +4°C and then fixed using Carnoy solution (3:1 absolute alcohol:glacial acetic acid) overnight. Fixed root tips were transferred to 70% alcohol and stored at +4°C until analysis. Afterwards, the root tips were hydrolyzed with 1 N HCl for 12 minutes at 60°C and stained with 2% aceto orcein for 24 hours at room temperature. Stained root tips were squashed in a drop of 45% acetic acid and the preparations were mounted in entellan in order to obtain permanent slides. The best metaphase plates, including at least ten well-spread cells, were photographed with an Olympus BX51 microscope with an attached digital camera, and they were also drawn from permanent slides. For chromosome counting, both the 10 × 100 enlarged photographs and the drawings were used (Jones & Rickards 1991; Elçi 1994; Martin et al. 2019).

**DNA isolation, PCR amplification, and sequencing**

Total genomic DNA was extracted from silica dried leaves following the modified CTAB extraction procedure of Doyle & Doyle (1987). The nrITS region and *trnT-trnL* spacer were amplified using the universal ‘ITS4 and ITSS’ primers (White et al. 1990) and the universal ‘a and b’ primers (Taberlet et al. 1991), respectively, according to the PCR conditions described by Gültepe et al. (2010). The PCR products were sequenced with the aid of Macrogen Inc. (Seoul, Korea) using the same primers.

**DNA sequence alignment and phylogenetic analyses**

The nucleotide sequences were aligned by ClustalW using BioEdit v.7.0 software (Hall 1999). The obtained sequence data were compared with GenBank sequences using BLAST and manually verified. For both ITS and *trnT-trnL* sequences, total nucleotide length (bp), parsimony informative sites and the interspecific and intraspecific pairwise differences from a distance matrix were calculated using MEGA v.7.0 (Kumar et al. 2016).
The phylogenetic analysis included 52 *Vincetoxicum* taxa attributed to the ‘*Vincetoxicum* s. str. clade’, as well as one accession attributed to the ‘Far Eastern clade’ according to Liede-Schumann *et al.* (2016). Of these, 31 accessions belonged to 10 taxa of Turkish *Vincetoxicum* s. str. (Appendix 1), and the dataset of ITS and *trn*T-*trn*L belonging to the remaining 22 accessions studied by Liede-Schumann *et al.* (2016) was retrieved from GenBank (www.ncbi.nlm.nih.gov). Our sampling aimed to cover the area of the genus *Vincetoxicum* s. str. in Turkey as completely as possible, with particular emphasis on the ‘*Vincetoxicum* s. str. clade’ including the taxa attributed to the European, Western Irano-Turanian, Eastern Mediterranean, Southern Russian, Eastern Irano-Turanian, and Western Himalayan clades (Liede-Schumann *et al.* 2016), which are neighbouring regions to Turkey. As outgroup, six specimens belonging to three different species of Asclepiadoideae (*Cionura erecta*, *Cynanchum acutum* and *Gomphocarpus fruticosus*), which are also distributed in Turkey, were selected for the phylogenetic analysis (Appendix 1).

The two dataset (ITS and *trn*T-*trn*L) were combined according to the ILD test results of Liede-Schumann *et al.* (2012). Phylogenetic relationships were reconstructed using Bayesian Inference (BI) analyses and Maximum Parsimony (MP). For Bayesian Inference (BI) analyses, evolutionary models were assessed with Akaike information criterion (AIC) as implemented in MrModeltest 2.3 software (Nylander 2004). The most suitable models, as detected by AIC, were GTR+G for the nrDNA ITS marker and GTR+I for the cpDNA *trn*T-*trn*L marker. The BI methods were carried out using MrBayes ver. 3.1 (Ronquist & Huelsenbeck 2003). For the BI analyses, a Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm was employed with two simultaneous runs of four parallel MCMCMC each for 1 million generations, starting with a random tree. The trees were saved every 1000th generation, and the first 20% were discarded as ‘burn-in’. The remaining trees were used to estimate the majority rule consensus tree and Bayesian posterior probabilities (PP).

Maximum Parsimony (MP) methods were implemented in the PAUP* ver. 4.0b10 (Swofford 2003) software. For the MP analyses, a script was first created using the parsimony ratchet (Nixon 1999) method implemented in the PRAP v.2.0 (Müller 2004). The script contains standard ratchet settings (200 ratchet iterations with 25% of the positions randomly upweighted (weight = 2) during each replicate and 10 random additional cycles). This file, containing the sequence data and commands, was analysed by Jackknife (JK) in PAUP* ver. 4.0b10 (Swofford 2003) to calculate JK support values for the branches. Then, MP analysis in PAUP* ver. 4.0b10 (Swofford 2003) was performed with 1000 repetitions according to the majority rule. The JK support values were transferred to the phylogenetic tree obtained from the results of the MP analysis. Indels were coded as informative characters according to the Simple Indel Coding (SIC) method (Simmons & Ochoterena 2000) as implemented in the programme SeqState ver. 1.40 (Müller 2005) and added at the end of the sequence dataset.

In phylogenetic analyses based on sequence data, monophyly, branch lengths, branch support and genealogical concordance have been reported as often-used criteria for species delimitation (Leliaert *et al.* 2014). Our phylogeny based on nrDNA (ITS) and cpDNA (*trn*T-*trn*L) sequences contains multiple specimens per species, and species are delimited based on topological criteria, monophyly and branch support. Additionally, some typical floral characteristics of *Vincetoxicum* s. str., which were not analysed, were also presented in our phylogenetic tree in order to see whether there is a concordance between molecular data and these diagnostic characteristics.

**Results**

**Chromosome numbers**

Chromosome counts for 16 accessions belonging to ten taxa of *Vincetoxicum* s. str. from Turkey are presented in Figs 1–2. The somatic chromosome numbers were determined as 2n=4x=44 in two subspecies...
Fig. 1. Somatic metaphase chromosome (1 = light microscope; 2 = outline drawing). a. *Vincetoxium canescens* (Willd.) Decne. subsp. *canescens* (Güven 36 & Makbul, 2n=22). b. *V. canescens* (Willd.) Decne. subsp. *pedunculata* Browicz (Güven 51 & Makbul, 2n=22). c. *V. funebre* Boiss. & Kotschy (Güven 126 & Makbul, 2n=22). d. *V. fuscatum* (Hornem.) Rchb. subsp. *boissieri* (Kusn.) Browicz (Güven 35 & Makbul, 2n=44). e. *V. fuscatum* (Hornem.) Rchb. subsp. *fuscatum* (Güven 93 & Makbul, 2n=44). Scale bars = 5 µm.
Fig. 2. Somatic metaphase chromosome (1 = light microscope; 2 = outline drawing). a. *Vincetoxicum hirundinaria* Medik. subsp. *hirundinaria* (Güven 135 & Makbul, 2n=22). b. *V. parviflorum* Decne. (Güven 80 & Makbul, 2n=22). c. *V. scandens* Sommier & Levier (Güven 30 & Makbul, 2n=22). d. *V. speciosum* Boiss. & Spruner (Güven 137 & Makbul, 2n=22). e. *V. tmoleum* Boiss. (Güven 72 & Makbul, 2n=22). Scale bars = 5 µm.
Table 1. List of specimens used for cytological studies.

| Taxa                     | Vouchers           | IUCN categories (Ekim et al. 2000) | Present chromosome counts | Previous records                  |
|--------------------------|--------------------|------------------------------------|---------------------------|-----------------------------------|
| *V. canescens*           | Güven 36 & Makbul  | –                                  | $2n = 2x = 22$            | $2n = 2x = 22$ (Li et al. 1995)   |
| subsp. canescens         |                    |                                    |                           |                                   |
| *V. canescens*           | Güven 51 & Makbul  | VU                                 | $2n = 2x = 22$            | –                                 |
| subsp. pedunculata       |                    |                                    |                           |                                   |
| *V. funebre*             | Güven 126 & Makbul| DD                                 | $2n = 2x = 22$            | –                                 |
| *V. fucatum*             | Güven 32 & Makbul, | LC (endemic)                       | $2n = 4x = 44$            | –                                 |
| subsp. boissieri         | Güven 35 & Makbul  |                                    |                           |                                   |
| *V. fucatum*             | Güven 23 & Makbul, | –                                  | $2n = 4x = 44$            | $2n = 2x = 22$ (Strid &          |
| subsp. fucatum           | Güven 77 & Makbul, |                                    |                           | Franzen 1981)                     |
|                         | Güven 93 & Makbul  |                                    |                           |                                   |
| *V. hirundinaria*        | Güven 18 & Makbul, | –                                  | $2n = 2x = 22$            | $2n = 2x = 22$ (Uhriková et al. |
| subsp. hirundinaria      | Güven 28 & Makbul, |                                    |                           | 1985; Liede-Schumann et al. 2012)|
|                         | Güven 135 & Makbul|                                    |                           |                                   |
| *V. parviflorum*         | Güven 80 & Makbul  | NT (endemic)                       | $2n = 2x = 22$            | $2n = 2x = 22$ (Uhríková et al.  |
| *V. scandens*            | Güven 30 & Makbul  | –                                  | $2n = 2x = 22$            | 1985; Liede-Schumann et al. 2012)|
| *V. speciosum*           | Güven 137 & Makbul|                                    | $2n = 2x = 22$            | $2n = 2x = 22$ (Pardi 1933)      |
| *V. tmoleum*             | Güven 67 & Makbul, | –                                  | $2n = 2x = 22$            | $2n = 2x = 22$ (Pardi 1933)      |
|                         | Güven 72 & Makbul  |                                    |                           |                                   |

of *V. fucatum* (subsp. fucatum and subsp. boissieri) and $2n = 2x = 22$ in the rest of the examined taxa. To the best of our knowledge, these are the first chromosome counts for *V. canescens* subsp. pedunculata, *V. funebre*, *V. fucatum* subsp. boissieri, *V. parviflorum* and *V. tmoleum* (Table 1).

DNA sequence alignments

For the 31 examined Turkish *Vincetoxicum* s. str. samples, the length of the amplified nrDNA ITS region was 617 or 618 bp. The gap-less alignment of 618 bp, included 594 (96.1%) constant, 24 (3.9%) variable and 23 (3.7%) parsimony informative sites. The estimated Transition/Transversion bias (R) was 3.69. The length of the *trnT-trnL* region was 804 bp. The dataset had an aligned length of 804 sites, of which 802 (99.8%) were constant, 2 (0.25%) were variable and 1 was (0.12%) parsimony informative. R was determined as 0.00.

Phylogenetic analyses

The topologies of the phylogenetic trees inferred from the concatenated alignment of nrDNA ITS + *trnT-trnL* using MP and BI analyses were similar. Therefore, the Bayesian majority rule tree is shown in Fig. 3, along with JK support values of the MP analysis. The phylogenetic tree revealed that all the examined taxa of *Vincetoxicum* were separated from the outgroup (which included specimens belonging to the three separate species: *Cionura erecta*, *Cynanchum acutum* and *Gomphocarpus fruticosus*) and formed a clade composed of two subclades, I and II. Clade I comprised only *V. atratum* corresponding to the ‘Far Eastern clade’ in Liede-Schumann et al. (2016), and clade II, corresponding to the ‘*Vincetoxicum* s. str. clade’ in Liede-Schumann et al. (2016), included all the remaining ingroup taxa. Our phylogenetic tree (Fig. 3) revealed that among the examined taxa of Turkish *Vincetoxicum* s. str., only *V. fucatum*,...
**Fig. 3.** Phylogenetic tree of *Vincetoxicum* s. str., including Turkish samples (in **bold**) based on the combined dataset (ITS and trnT-trnL). The support values on branches indicate Jackknife (JK) and Posterior Probability (PP) higher than 50% and 0.7, respectively. Clade designations correspond to Liede-Schumann et al. (2016). Refer to Appendix 1 for accession abbreviations (G:Güven, M:Makbul).

*V. parviflorum* and *V. speciosum* were recovered as monophyletic, whereas the delimitation of the remaining six taxa was less clear based on molecular data. In the phylogenetic tree, some typical floral characteristics of the investigated taxa of *Vincetoxicum* s. str. such as the indumentum of the corolla and shape of the corona (which were not analysed) were also presented. Nevertheless, these characters seemed to be homoplastic, since they appeared in different subclades.

**Discussion**

In the present study, the chromosome number for ten taxa of *Vincetoxicum* s. str. from Turkey, including two endemics, are reported. In addition, the phylogenetic positions of *V. fuscatum* subsp. *boissieri* and *V. parviflorum*, which are endemic to Turkey, were investigated based on nrDNA ITS and cpDNA trnT-trnL with 31 newly generated sequences.
In the Flora of Turkey (Browicz 1975), the yellow flowered *V. canescens* was represented by two subspecies, subsp. *canescens* and subsp. *pedunculata*, which were shown in the present study to be diploid according to two Turkish accessions. The chromosome number of the other yellowish flowered Turkish *Vincetoxicum s.str.*, *V. tmoleum*, was determined as $2n=2x=22$ from two Turkish accessions. To the best of our knowledge, these are the first chromosome counts for *V. canescens* subsp. *pedunculata* and *V. tmoleum*. Similarly, *V. canescens* subsp. *canescens* has previously been reported as diploid ($2n=2x=22$) in the Flora of China (Li *et al.* 1995). Our chromosomal data on *V. canescens* subsp. *canescens* originating from Turkey is in accordance with these previous results.

According to our phylogenetic analyses, *V. canescens* and *V. tmoleum* also clustered in the same group (including *V. creticum*) corresponding to the ‘Eastern Mediterranean clade’ in Liede-Schumann *et al.* (2016). It was reported that these two species had many morphological and palynological characteristics in common, such as densely hairy corolla and deeply parted corona segments (Güven 2017), an ovate pollinium and corpusculum shape, and a rugulate pollen surface (Güven *et al.* 2015). The similarity in floral morphology between *V. canescens* and *V. tmoleum* was also reported by Browicz (1975). In addition, the same chromosome number was determined for *V. canescens* and *V. tmoleum*. However, the stem and fruit morphology of the two species are quite different. *Vincetoxicum canescens*, characterised by decumbent stems with a canescent-tomentose indumentum and ovoid fruits, is easily distinguished from *V. tmoleum*, which has erect and crisped hairy stems and slender ovoid fruits (Browicz 1975; Güven 2017). *Vincetoxicum creticum* (endemic to Crete-Greece) clustered with *V. tmoleum* in the same sub-clade. This species was previously reported as morphologically close to *V. tmoleum* by Browicz (1975). Our molecular results support this view.

Two subspecies of *V. canescens*, subsp. *canescens* and subsp. *pedunculata*, differing from each other by the length of their peduncle (Browicz 1978), are closely related taxa in some anatomical and palynological aspects (Güven 2017). Our analyses showed that these two subspecies had identical chromosome numbers of $2n=22$ (diploid), and had identical sequences. Further molecular studies involving more gene regions will be needed to resolve the identity of the two subspecies.

*Vincetoxicum fuscatum* was represented by two morphologically close subspecies, subsp. *boissieri* and subsp. *fuscatum*, distinguished only by the corolla indumentum in the Flora of Turkey (Browicz 1975). Our analyses show that the two subspecies are tetraploid. While this was the first chromosome count for the subsp. *boissieri*, the chromosome number of $2n=22$ (diploid) has been reported for the subsp. *fuscatum* from Greece (Strid & Franzen 1981). The intraspecific differentiation of the ploidy level within the plant species might be a result of the geographical distribution of the taxa (Morawetz 1984). In support of this, it has been reported that plant taxa collected from various geographical regions can have different chromosome numbers (Ozcan *et al.* 2008). Similarly, the chromosome number of *V. nigrum* from Spain has been reported as $2n=2x=22$ (diploid) (Aparicio & Silvestre 1985), whereas it was counted as $2n=4x=44$ (tetraploid) in populations distributed in both the Netherlands (Van den Brand *et al.* 1979) and Canada (Liede-Schumann *et al.* 2012). These differences can be caused by the variations in populations of *V. nigrum* and in environmental conditions between different geographical regions.

The taxa of *V. fuscatum* clustered under the same subclade together with *V. maeoticum* from Russia and *V. intermedium* from Kazakhstan (Fig. 3), corresponding to the ‘Southern Russian clade’ of Liede-Schumann *et al.* (2016). Marhold (2011) reported that *V. maeoticum* and *V. intermedium* are probably synonyms of *V. fuscatum* subsp. *fuscatum*. This view is supported by our molecular results. Browicz (1978) recognised that *V. fuscatum* subsp. *boissieri* and *V. fuscatum* subsp. *fuscatum*, which were distinguished only by their corolla indumentum, were separated at the subspecies level based on high morphological similarities. However, Pobedimova (1952) previously treated these two taxa as separate
species named Antitoxicum boissieri (Kusn.) Pobed. (= V. fuscatum subsp. boissieri) and Antitoxicum minus (K.Koch) Pobed. (= V. fuscatum subsp. fuscatum). Our karyological analyses showed that these two subspecies of V. fuscatum had identical chromosome numbers of 2n=44 (tetraploid). Otherwise, in our molecular analyses, three specimens of subsp. boissieri formed a well-supported subclade among the unresolved specimens of V. fuscatum subsp. fuscatum, V. maeoticum and V. intermedium. We therefore suggest to raise V. fuscatum subsp. boissieri to the species level sister to V. fuscatum according to the present molecular data.

The somatic chromosome number of V. hirundinaria subsp. hirundinaria, which was the first report from Turkish accessions, was determined as 2n=2x=22. Our results are consistent with the previous counts reported by Uhríková et al. (1985) in Slovakia and Liede-Schumann et al. (2012) in Germany. The present study revealed a diploid chromosome number of 2n=2x=22 for V. scandens and V. speciosum from Turkish accessions. These findings are in agreement with the previous results of V. speciosum in Iran (Lessani & Chariat-Panahi 1979) and V. speciosum in Italy (Pardi 1933). In this study, the chromosome number of V. funebræ was determined for the first time as 2n=2x=22 from a Turkish accession.

Vincetoxicum hirundinaria subsp. hirundinaria, characterised by white-flowers, clustered with V. jailicola, V. rossicum and V. schmalhausenii (the European clade, according to Liede-Schumann et al. 2016), forming a clade in sister-group position to V. funebræ, V. rehmannii and V. scandens (the Irano-Turanian clade according to Liede-Schumann et al. 2016), as well as to V. speciosum. The present molecular results are in accordance with the chromosome counts as well as some morphological and palynological features of individuals of the four Turkish Vincetoxicum taxa (V. funebræ, V. hirundinaria subsp. hirundinaria, V. scandens and V. speciosum). Güven (2017) also reported that these taxa were characterised by a densely or sparsely hairy corolla with cup-shaped corona and obovate pollinia.

Vincetoxicum scandens, characterised by twining stems up to 2 m high, clustered with V. funebræ, which is known to have erect and shorter (40–135 cm) stems. However, these taxa have some floral features in common, such as hairy corolla and cup-shaped five-parted corona (Pobedimova 1952; Güven 2017). Furthermore, V. funebræ and V. scandens have similar palynological (obovate pollinium and oblong corpusculum) characteristics (Güven et al. 2015) and the same chromosome number of 2n=2x=22.

Vincetoxicum speciosum accessions formed a distinct subclade. In Turkey, V. hirundinaria subsp. hirundinaria and V. speciosum share the same overlapping humid habitats such as Quercus-forest openings in northwest Anatolia and Thrace regions. However, these two diploid representatives of Vincetoxicum can easily be differentiated by their morphological and palynological characteristics. While V. speciosum is characterised by a blackish purple corolla and an erect stem with wholly velutinous pubescence, V. hirundinaria subsp. hirundinaria has a white corolla and crisped hairy stems that occasionally twine near the apex (Browicz 1975). Güven et al. (2015) noted that the pollinia surface of V. speciosum exhibited gemmate ornamentation, in contrast to the rugulate surface in V. hirundinaria subsp. hirundinaria. Furthermore, V. speciosum was characterised by the longest obovate pollinium among the Turkish Vincetoxicum s. str. taxa (Güven et al. 2015).

Vincetoxicum nigrum is characterised by a weak twining stem and dark blackish-purple coloured and hairy flowers. Browicz (1978) indicated that the records previously reported from Anatolia for V. nigrum were incorrect and so they were treated under V. scandens. Vincetoxicum nigrum is morphologically similar to V. scandens but differs from this species by its weak growth, smaller leaves, shorter peduncles (not more than 1 cm), and cup-shaped corona with small coronal teeth (Markgraf 1972; Browicz 1978). Additionally, while both di- (2n=22) and tetraploid (2n=44) chromosome numbers have been counted for V. nigrum (Liede-Schumann et al. 2012), the chromosome counts for V. scandens, reported in both
the present and previous (Lessani & Chariat-Panahi 1979) studies, were all 2n=22 (diploid). These two species are also located in different subclades according to the present molecular results.

In the present study, the chromosome number of *V. parviflorum* was determined for the first time as 2n=2x=22 from a Turkish accession. In the Flora of Turkey, Browicz (1978) reported that *V. parviflorum* could be a small-flowered variety of *V. fuscatum* due to their similar flowers and habits. However, Boissier (1875), who treated *V. fuscatum* and *V. parviflorum* as different at the species level, noted that these two taxa differ from each other in a number of morphological characters related to the stem and the flower. In accordance with Boissier (1875), Güven et al. (2015) also determined that although *V. parviflorum* exhibited an elliptic pollinium, two subspecies of *V. fuscatum* were characterised by a clavate pollinium. While the somatic chromosome number was determined as 2n=22 (diploid) in *V. parviflorum*, it was counted as 2n=44 (tetraploid) for both subspecies of *V. fuscatum*. Our molecular analysis also showed that *V. parviflorum* separated from taxa of *V. fuscatum* and clustered in a different subclade with *V. assadii*, *V. mozaffarianii* and *V. pumilum* (Eastern Irano-Turanian taxa according to Liede-Schumann et al. (2016)).

In conclusion, the results of our phylogenetic analyses, including 31 newly generated ITS and *trnT-trnL* sequences belonging to 10 taxa of *Vincetoxicum* s. str. from Turkey, agrees largely with previous phylogenetic results (Liede-Schumann et al. 2016). In addition to the previous study, DNA sequences were determined for *V. fuscatum* subsp. *boissieri* and *V. parviflorum* endemic to Turkey, as well as *V. speciosum* of European origin. Of the studied taxa, *V. fuscatum*, *V. parviflorum* and *V. speciosum* are clearly resolved at the species level, whereas the delimitation of the rest of the Turkish taxa was less clear based on molecular data. Three specimens of the endemic *V. fuscatum* subsp. *boissieri* formed a well-supported subclade within the clade including unresolved specimens of *V. fuscatum* subsp. *fuscatum*, *V. maecticum* and *V. intermedium*. We therefore suggest that *V. fuscatum* subsp. *boissieri*, previously treated as *Antitoxicum boissieri* by Pobedimova (1952), should be raised to the species level, sister to *V. fuscatum*. The other endemic taxon *V. parviflorum*, reported as a morphologically related taxon to *V. fuscatum* by Browicz (1978), separated from the *V. fuscatum* taxa and clustered in a different clade, sister to *V. assadii*, *V. mozaffarianii* and *V. pumilum* from Iran. The molecular results were evaluated together with some typical floral characteristics of *Vincetoxicum* s. str., such as the indumentum of the corolla and shape of the corona (which were not analysed). Nevertheless, these characters seemed to be homoplastic, since they appeared in different subclades.

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Appendix 1. Locality information, voucher specimens and accession numbers for sequences in GenBank (NCBI) of the examined specimens used for molecular (Mol.) and cytological (Cyt.) studies. Sequences previously published are indicated in brackets (A=Liede-Schumann et al. 2016, B=Liede-Schumann et al. 2012, C=Liede et al. 2002, D=Liede 2001, E=Berner & Carter Unpublished, F=Lahaye et al. 2005, G=Liede & Täuber 2002, H=Goyder et al. 2007, PS=present study).

| Taxon                                      | Taxon voucher, herbarium, origin                                      | GenBank accession number (Mol.)                 | Cyt. |
|--------------------------------------------|---------------------------------------------------------------------|------------------------------------------------|------|
| **Ingroup**                                |                                                                     | ITS (ITS1, 5.8S rRNA gene, ITS2)               |      |
| *Vincetoxicum arnottianum* (Wight) Wight   | Rizwana s.n., PMNH, Pakistan                                        | LN880574.1 (A)                                |      |
|                                            |                                                                     | LN880797.1 (A)                                |      |
| *Vincetoxicum assadii* Zaeifi               | Assadi et Abulharmzeh 46397, TARI, Iran                            | LN880575.1 (A)                                |      |
|                                            |                                                                     | LN880798.1 (A)                                |      |
| *Vincetoxicum atratum* (Bunge)              | Chen et al. 960287, MO, China                                       | HE793927.1 (B)                                |      |
|                                            |                                                                     | HE793734.1 (B)                                |      |
| *Vincetoxicum canescens* (Willd.) Decne.    |                                                                     | LN880577.1 (A)                                |      |
|                                            |                                                                     | LN880800.1 (A)                                |      |
| *Vincetoxicum canescens* (Willd.) Decne. subsp. canescens | Güven 58 & Makbul, RUB, Ankara: Beypare, İnozü valley, Turkey       | MN106189 (PS)                                 |      |
|                                            |                                                                     | MN159561 (PS)                                 |      |
|                                            |                                                                     | MN106188 (PS)                                 |      |
|                                            |                                                                     | MN159560 (PS)                                 | +    |
|                                            |                                                                     | MN106190 (PS)                                 |      |
|                                            |                                                                     | MN159562 (PS)                                 |      |
| *Vincetoxicum canescens* (Willd.) Decne. subsp. pedunculata Browicz | Güven 50 & Makbul, RUB, İzmir: Ödemiş, Bozdağ, Turkey               | MN106191 (PS)                                 |      |
|                                            |                                                                     | MN159563 (PS)                                 |      |
|                                            |                                                                     | MN106192 (PS)                                 |      |
|                                            |                                                                     | MN159564 (PS)                                 | +    |
|                                            |                                                                     | MN106193 (PS)                                 |      |
|                                            |                                                                     | MN159565 (PS)                                 |      |
| *Vincetoxicum creticum* Browicz             | Hilger s.n., UBT, Greece-Crete                                     | HE793928.1 (B)                                |      |
|                                            |                                                                     | HE793736.1 (B)                                |      |
| *Vincetoxicum funebre* Boiss. & Kotschy     | Pahlente an et Asef 20, IRAN, Iran                                 | LN880579.1 (A)                                |      |
|                                            |                                                                     | LN880802.1 (A)                                |      |
|                                            |                                                                     | MN106194 (PS)                                 |      |
|                                            |                                                                     | MN159566 (PS)                                 |      |
| *Vincetoxicum fuscatum* (Hornem.) Rchb. subsp. boissieri (Kusn.) Browicz | Güven 27 & Makbul, RUB, Gümüşhane: Torul, Turkey                    | MN106195 (PS)                                 |      |
|                                            |                                                                     | MN159567 (PS)                                 |      |
|                                            |                                                                     | MN106196 (PS)                                 |      |
|                                            |                                                                     | MN159568 (PS)                                 | +    |
|                                            |                                                                     | MN106197 (PS)                                 |      |
|                                            |                                                                     | MN159569 (PS)                                 |      |
| *Vincetoxicum fuscatum* (Hornem.) Rchb. subsp. fuscatum | Güven 32 & S. Makbul, RUB, Gümüşhane: Kelkit-Erzincan road, Turkey | –                                              | +    |
|                                            |                                                                     | –                                              |      |
|                                            |                                                                     | –                                              |      |
|                                            |                                                                     | –                                              |      |
| *Vincetoxicum fuscatum* (Hornem.) Rchb. subsp. fuscatum | Güven 22 & Makbul, RUB, İstanbul: Paşaköy, Ömerli Dam, Turkey       | MN106198 (PS)                                 |      |
|                                            |                                                                     | MN159570 (PS)                                 |      |
|                                            |                                                                     | MN106201 (PS)                                 |      |
|                                            |                                                                     | MN159573 (PS)                                 |      |
|                                            |                                                                     | MN106199 (PS)                                 |      |
|                                            |                                                                     | MN159571 (PS)                                 | +    |
|                                            |                                                                     | MN106202 (PS)                                 |      |
|                                            |                                                                     | MN159574 (PS)                                 |      |
### Appendix 1 (cont.)

| Taxon                     | Taxon voucher, herbarium, origin                                                                                                                                                                                                 | GenBank accession number (Mol.) | Cyt. |
|---------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|------|
| *Vincetoxicum fuscatum* (Hornem.) Rehb. subsp. *fuscatum* (cont.)                       | Güven 93 & Makbul, RUB, Niğde: Ulukışla, Maden Village, Turkey                                                                                                                                   | MN106200 (PS)  MN159572 (PS) + | +   |
|                            | Güven 77 & Makbul, RUB, Tunceli: Pülümür, Turkey                               | MN106201 (PS)  MN159573 (PS) + | +   |
| *Vincetoxicum glaucum* (Wall. ex Wight) Rech.f.                                      | Jan Alam 14963, KUH, Pakistan                                                                                                                                         | LN880581.1 (A)  LN880805.1 (A) | +   |
| *Vincetoxicum hirundinaria* Medik. subsp. *hirundinaria*                              | Güven 28 & Makbul, RUB, Kırklareli: Demirköy-İğneada road, Turkey                                                                                           | MN106204 (PS)  MN159576 (PS) + | +   |
|                            | Güven 18 & Makbul, RUB, Kırklareli: Vize, Kömürköy, Turkey                                                                                                           | MN106203 (PS)  MN159575 (PS) + | +   |
| *Vincetoxicum hirundinaria* Medik.                                                    | Meve 970, UBT, Germany                                                                                                                                                    | AJ320474.1 (C)  AJ410274.1 (D) |      |
| *Vincetoxicum intermedium* Taliev                                                     | Cherkasova s.n., MW, Kazakhstan                                                                                                                                          | LN880583.1 (A)  LN880807.1 (A) |      |
| *Vincetoxicum jailicola* Juz.                                                        | Vylegzeanina s.n., MW, Russia                                                                                                                                             | LN880584.1 (A)  LN880808.1 (A) |      |
| *Vincetoxicum maeoticum* (Kleopow) Barbar.                                            | Kopylov-Guskov et al. s.n., MW, Russia                                                                                                                                       | LN880586.1 (A)  LN880810.1 (A) |      |
| *Vincetoxicum mozaffarianii* Zaeifi                                                   | Mozaffarian 4479, TARI, Iran                                                                                                                                              | LN880587.1 (A)  LN880811.1 (A) |      |
| *Vincetoxicum nigraum* (L.) Moench                                                   | New York                                                                                                                                                              | FJ362532.1 (E)  AY899964.1 (F) |      |
|                            | Çiveyrel 1106                                                                                                                                                    | MN106207 (PS)  MN159579 (PS) + | +   |
|                            | Güven 95 & Makbul, RUB, Kayseri: Yahyali, Kapuzbaşı Waterfalls, Turkey                                                                                                         | MN106206 (PS)  MN159578 (PS) + |      |
| *Vincetoxicum parviflorum* Decne.                                                    | Güven 80 & Makbul, RUB, Tunceli: Ovacık, Karagöl Valley, Turkey                                                                                                      | MN106207 (PS)  MN159579 (PS) + | +   |
|                            | Güven 101 & Makbul, RUB, Ordu: Ünye, İnkur-Akkuş road, Turkey                                                                                                         | MN106209 (PS)  MN159581 (PS) |      |
| *Vincetoxicum persicum* Decne.                                                       | Djavadi et al. 56807, IRAN                                                                                                                                           | MN106208 (PS)  MN159580 (PS) + | +   |
| *Vincetoxicum rossicum* (Kleopow) Barbar.                                             | isolate CYKVI-4, New York                                                                                                                                              | FJ517165.1 (E)  HE793742.1 (B) |      |
|                            | Murray s.n., UBT, Canada                                                                                                                                            |                     |      |
| *Vincetoxicum rehmannii* Boiss.                                                      | Schoenswetter et Tribsch 21,W, Georgia                                                                                                                                 | LN880590.1 (A)  LN880814.1 (A) |      |
| *Vincetoxicum sakesarensense* Ali & Khatoon                                          | Rizwana 036586, PMNH, Pakistan                                                                                                                                         | LN880592.1 (A)  LN880816.1 (A) |      |
| *Vincetoxicum scandens* Somnier & Levier                                              | Pahlevani et Asef 27, IRAN 54575, Iran                                                                                                                                 | MN106204 (PS)  MN159580 (PS) + | +   |
### Vincetoxicum scandens Sommier & Levier
- Güven 30 & Makbul, RUB, Rize: Çamlıkköy Village, Turkey
- Güven 121 & Makbul, RUB, Artvin: Boçka, Uğur Village, Turkey

### Vincetoxicum schmalhausenii (Kusn.) Litv.
- Zernov s.n., MW, Russia

### Vincetoxicum speciosum Boiss. & Spruner
- Güven 137 & Makbul, RUB, Kırklareli: Demirköy, Mahya Mount, Turkey
- Güven 43 & Makbul, RUB, Zonguldak: Çaycuma-Zongudak road, Turkey
- Güven 112 & Makbul, RUB, Sinop: İnceburun, Turkey
- Güven 131 & Makbul, RUB, Bursa: İnegöl, Sayfiye Village, Turkey

### Vincetoxicum stocksii Ali & Khatoon
- S.I. Ali & S. Khatoon s.n., Pakistan
- Ali & Khatoon s.n., GA, Pakistan

### Vincetoxicum tauricum Pobed.
- Shvedehikova s.n., MW, Russia

### Vincetoxicum tmoleum Boiss.
- Ehrendorfer et al. 787-84-6, W, Turkey
- Güven 60 & Makbul, RUB, Ankara: Beypazarı, Karagöl Plateau, Turkey
- Güven 123 & Makbul, RUB, Artvin: Şavşat, Meydancık Gateway, Turkey
- Güven 48 & Makbul, RUB, Manisa: Salihli-Bozdağ road, Turkey
- Güven 75 & Makbul, RUB, Hatay: Amanos Mountains, Turkey
- Güven 67 & Makbul, RUB, Artvin: Arvin-Şavşat road, Turkey
- Güven 72 & Makbul, RUB, Erzurum: Oltu-Erzurum road, Turkey

### Outgroup

### Cionura erecta L.
- Güven 44 & Makbul, RUB, Zonguldak: Bakacakkadi, Turkey
- Güven 54 & Makbul, RUB, Denizli: Honaz Mountain, Turkey
- Güven 92 & Makbul, RUB, Adana: Pozantı-Ulukışla road, Turkey

### Cynanchum acutum L.
- Güven 140 & Makbul, RUB, Kırklareli: İğneada, Turkey

### Gomphocarpus fruticosus (L.) W.T.Aiton
- Nicholas 2796, UDW, South Africa: Eastern Cape

| Taxon | Taxon voucher, herbarium, origin | GenBank accession number (Mol) | Cyt.
|-------|----------------------------------|-------------------------------|------|
| Vincetoxicum scandens | Güven 30 & Makbul, RUB, Rize: Çamlıkköy Village, Turkey | MN106208 (PS), MN159580 (PS) | + |
| Vincetoxicum schmalhausenii | Zernov s.n., MW, Russia | LN880595.1 (A), LN880819.1 (A) | |
| Vincetoxicum speciosum | Güven 137 & Makbul, RUB, Kırklareli: Demirköy, Mahya Mount, Turkey | MN106214 (PS), MN159586 (PS) | + |
| Vincetoxicum stocksii | S.I. Ali & S. Khatoon s.n., Pakistan | AJ320475.1 (C) | |
| Vincetoxicum tauricum | Shvedehikova s.n., MW, Russia | LN880596.1 (A), LN880820.1 (A) | |
| Vincetoxicum tmoleum | Ehrendorfer et al. 787-84-6, W, Turkey | LN880597.1 (A), LN880821.1 (A) | |
| Cionura erecta | Güven 44 & Makbul, RUB, Zonguldak: Bakacakkadi, Turkey | MN106219 (PS), MN159591 (PS) | |
| Cynanchum acutum | Nicholas 2796, UDW, South Africa: Eastern Cape | AM396906.1 (H), AM295764.1 (H) | |
| Outgroup | | | |