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Research article

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

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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 (2*n*=44) and the remaining eight taxa diploid (2*n*=22). Molecular phylogenetic analyses based on nrDNA (ITS) and cpDNA (*trn*T-*trn*L) (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 (Ilçim *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 *trn*T-*trn*L spacer were amplified using the universal 'ITS4 and ITS5' 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 *trn*T-*trn*L 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

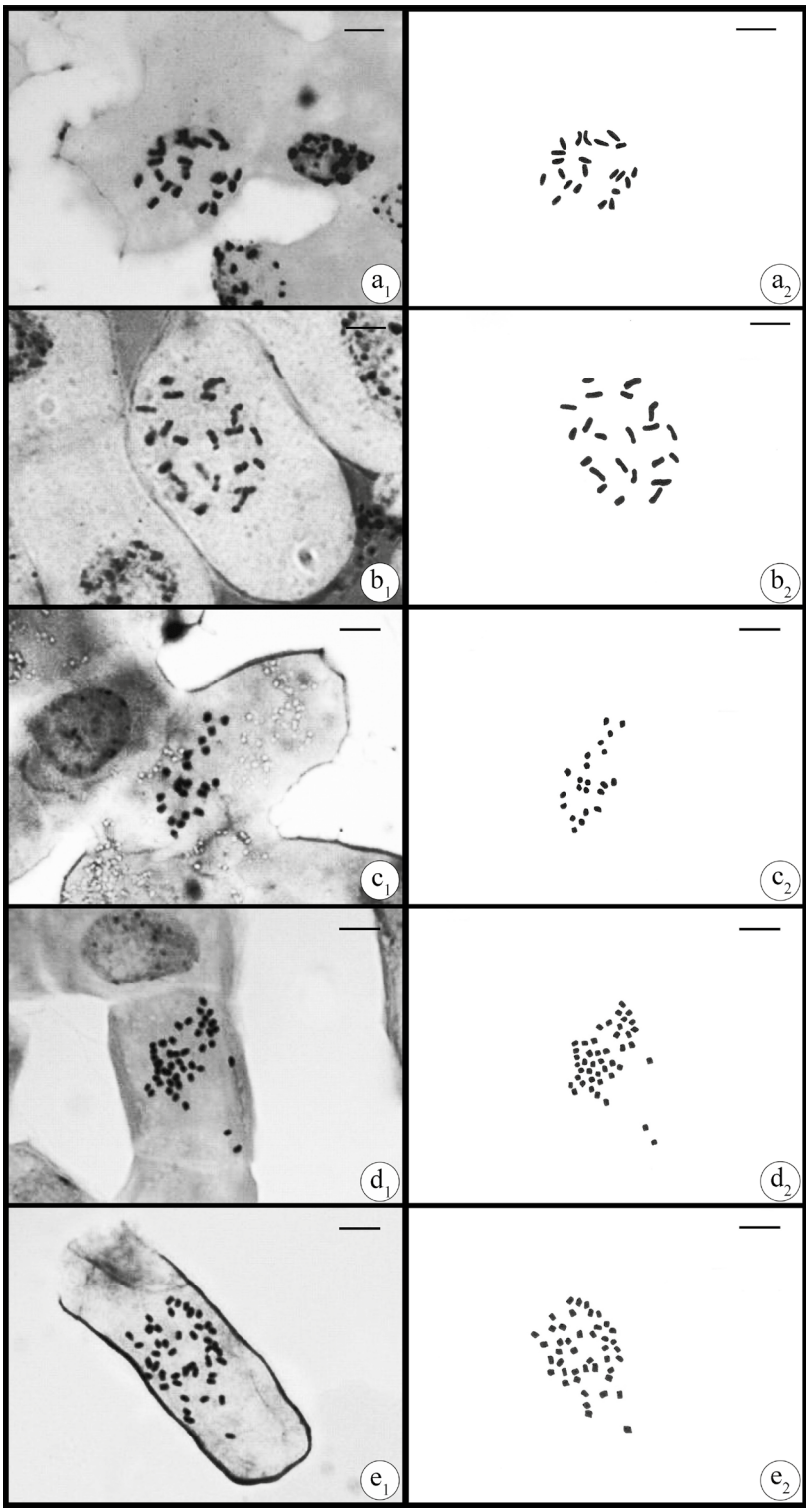

Fig. 1. Somatic metaphase chromosome (1 = light microscope; 2 = outline drawing). **a**. *Vincetoxium canescens* (Willd.) Decne. subsp. *canescens* (Güven 36 & Makbul, 2*n*=22). **b**. *V. canescens* (Willd.) Decne. subsp. *pedunculata* Browicz (Güven 51 & Makbul, 2*n*=22). **c**. *V. funebre* Boiss. & Kotschy (Güven 126 & Makbul, 2*n*=22). **d**. *V. fuscatum* (Hornem.) Rchb. subsp. *boissieri* (Kusn.) Browicz (Güven 35 & Makbul, 2*n*=44). **e**. *V. fuscatum* (Hornem.) Rchb. subsp. *fuscatum* (Güven 93 & Makbul, 2*n*=44). Scale bars = 5 µm.

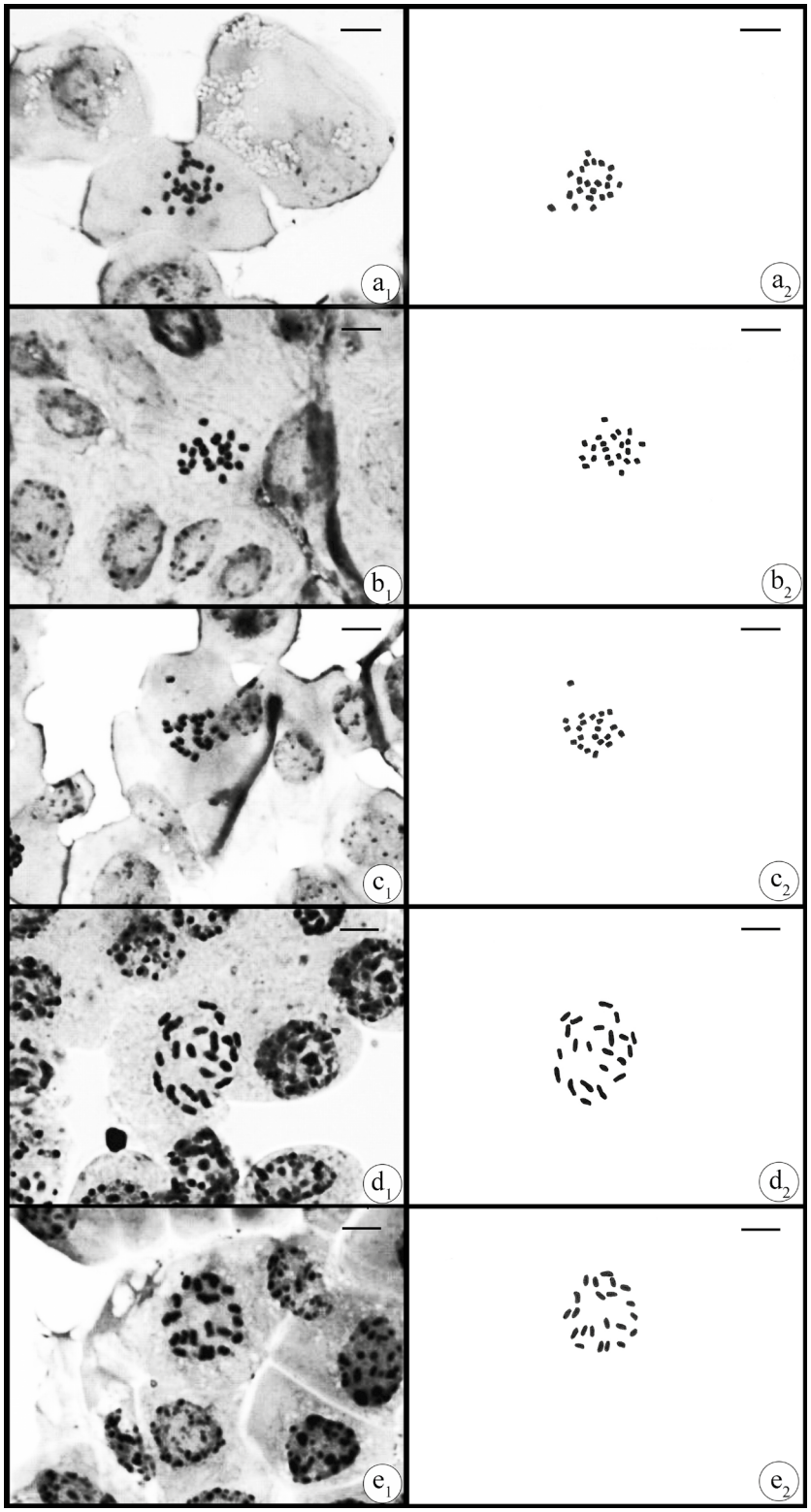

Fig. 2. Somatic metaphase chromosome (1 = light microscope; 2 = outline drawing). **a**. *Vincetoxicum hirundinaria* Medik. subsp. *hirundinaria* (Güven 135 & Makbul, 2*n*=22). **b**. *V. parviflorum* Decne. (Güven 80 & Makbul, 2*n*=22). **c**. *V. scandens* Sommier & Levier (Güven 30 & Makbul, 2*n*=22). **d**. *V. speciosum* Boiss. & Spruner (Güven 137 & Makbul, 2*n*=22). **e**. *V. tmoleum* Boiss. (Güven 72 & Makbul, 2*n*=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* subsp. *canescens	Güven 36 & Makbul	–	2*n*=2*x*=22	2*n*=2*x*=22 (Li *et al*. 1995)
V. canescens* subsp. *pedunculata	Güven 51 & Makbul	VU	2*n*=2*x*=22	–
V. funebre	Güven 126 & Makbul	DD	2*n*=2*x*=22	–
V. fuscatum* subsp. *boissieri	Güven 32 & Makbul, Güven 35 & Makbul	LC (endemic)	2*n*=4*x*=44	–
V. fuscatum* subsp. *fuscatum	Güven 23 & Makbul, Güven 77 & Makbul, Güven 93 & Makbul	–	2*n*=4*x*=44	2*n*=2*x*=22 (Strid & Franzen 1981)
V. hirundinaria* subsp. *hirundinaria	Güven 18 & Makbul, Güven 28 & Makbul, Güven 135 & Makbul	–	2*n*=2*x*=22	2*n*=2*x*=22 (Uhríková *et al*. 1985; Liede-Schumann *et al*. 2012)
V. parviflorum	Güven 80 & Makbul	NT (endemic)	2*n*=2*x*=22	–
V. scandens	Güven 30 & Makbul	–	2*n*=2*x*=22	2*n*=2*x*=22 (Lessani & Chariat-Panahi 1979)
V. speciosum	Güven 137 & Makbul		2*n*=2*x*=22	2*n*=2*x*=22 (Pardi 1933)
V. tmoleum	Güven 67 & Makbul, Güven 72 & Makbul	–	2*n*=2*x*=22	–

of *V. fuscatum* (subsp. *fuscatum* and subsp. *boissieri*) and 2*n*=2*x*=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. fuscatum* subsp. *boissieri, V. parviflorum* and *V. tmolem* (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 *trn*T-*trn*L 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 + *trn*T-*trn*L 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. fuscatum*,

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 *trn*T-*trn*L with 31 newly generated sequences.

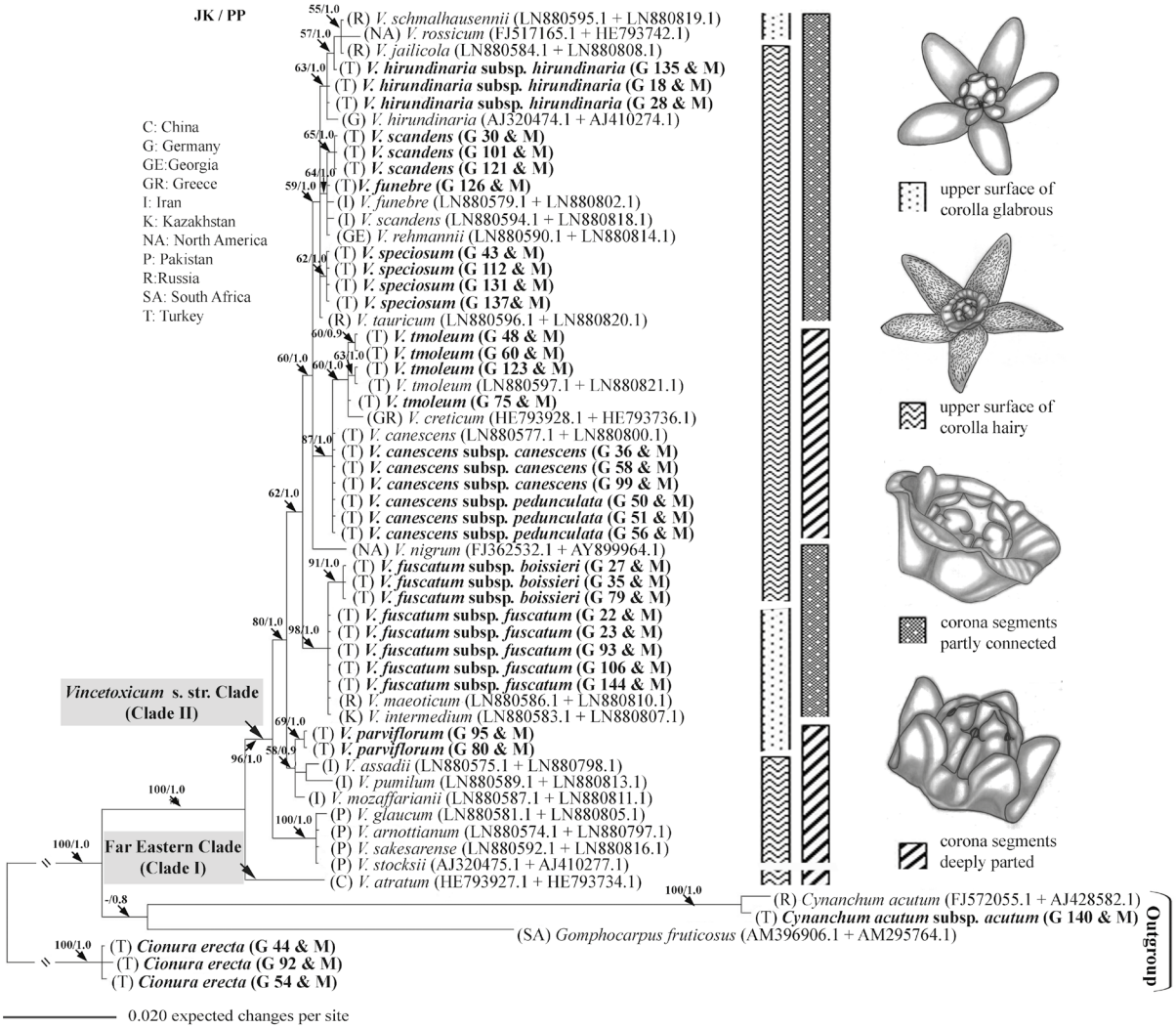

Fig. 3. Phylogenetic tree of *Vincetoxicum* s. str., including Turkish samples (in **bold**) based on the combined dataset (ITS and *trn*T-*trn*L). The support values on branches indicate Jackknife (JK) and Posterior Probability (PP) higher than 50% and 0.7, respectively. Clade designations correspond to Liede-Schumann *et al*. (2016). Refer to Appendix 1 for accession abbreviations (G:Güven, M:Makbul).

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 2*n*=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 2*n*=2*x*=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 2*n*=2*x*=22 for *V. scandens* and *V. speciosum* from Turkish accessions. These findings are in agreement with the previous results of *V. scandens* in Iran (Lessani & Chariat-Panahi 1979) and *V. speciosum* in Italy (Pardi 1933). In this study, the chromosome number of *V. funebre* was determined for the first time as 2*n*=2*x*=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. funebre*, *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. funebre*, *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. funebre*, 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. funebre* and *V. scandens* have similar palynological (obovate pollinium and oblong corpusculum) characteristics (Güven *et al*. 2015) and the same chromosome number of 2*n*=2*x*=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- (2*n*=22) and tetraploid (2*n*=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 2*n*=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 2*n*=2*x*=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 2*n*=22 (diploid) in *V. parviflorum*, it was counted as 2*n*=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 *trn*T-*trn*L 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. maeoticum* 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.
		ITS (ITS1, 5.8S rRNA gene, ITS2)	*trn*T-*trn*L	
Ingroup				
Vincetoxicum arnottianum (Wight) Wight	Rizwana s.n., PMNH, Pakistan	LN880574.1 (A)	LN880797.1 (A)	
Vincetoxicum assadii Zaeifi	Assadi et Abuhamzeh 46397, TARI, Iran	LN880575.1 (A)	LN880798.1 (A)	
Vincetoxicum atratum (Bunge) C.Morren & Decne.	Chen *et al*. 960287, MO, China	HE793927.1 (B)	HE793734.1 (B)	
Vincetoxicum canescens (Willd.) Decne.	s.coll. s.n, STU, Turkey	LN880577.1 (A)	LN880800.1 (A)	
Vincetoxicum canescens (Willd.) Decne. subsp. *canescens*	Güven 58 & Makbul, RUB, Ankara: Beypazarı, İnözü valley, Turkey	MN106189 (PS)	MN159561 (PS)	
	Güven 36 & Makbul, RUB, Erzincan: Kemah, Turkey	MN106188 (PS)	MN159560 (PS)	+
	Güven 99 & Makbul, RUB, Adana: Saimbeyli-Tufanbeyli road, Turkey	MN106190 (PS)	MN159562 (PS)	
Vincetoxicum canescens (Willd.) Decne. subsp. *pedunculata* Browicz	Güven 50 & Makbul, RUB, İzmir: Ödemiş, Bozdağ, Turkey	MN106191 (PS)	MN159563 (PS)	
	Güven 51 & Makbul, RUB, Manisa: Salihli-Kula road, Turkey	MN106192 (PS)	MN159564 (PS)	+
	Güven 56 & Makbul, RUB, Muğla: Yılanlı Mountain, Turkey	MN106193 (PS)	MN159565 (PS)	
Vincetoxicum creticum Browicz	Hilger s.n., UBT, Greece:Crete	HE793928.1 (B)	HE793736.1 (B)	
Vincetoxicum funebre Boiss. & Kotschy	Pahlenvani et Asef 20, IRAN, Iran	LN880579.1 (A)	LN880802.1 (A)	
	Güven 126 & Makbul, RUB, Ardahan: Posof, Erin Village, Turkey	MN106194 (PS)	MN159566 (PS)	+
Vincetoxicum fuscatum (Hornem.) Rchb. subsp. *boissieri* (Kusn.) Browicz	Güven 27 & Makbul, RUB, Gümüşhane: Torul, Turkey	MN106195 (PS)	MN159567 (PS)	
	Güven 35 & Makbul, RUB, Erzincan: Sakaltutan Gateway, Turkey	MN106196 (PS)	MN159568 (PS)	+
	Güven 79 & S. Makbul, RUB, Tunceli: Ovacık, Karagöl valley, Turkey	MN106197 (PS)	MN159569 (PS)	
	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)	
	Güven 106 & Makbul, RUB, Tokat: Keçeci Village, Turkey	MN106201 (PS)	MN159573 (PS)	
	Güven 23 & Makbul, RUB, Kütahya: Gediz, Turkey	MN106199 (PS)	MN159571 (PS)	+
	Güven 144 & Makbul, RUB, Sivas: İmranlı, Karaboğaz Village, Turkey	MN106202 (PS)	MN159574 (PS)	

Appendix 1 (cont.)

Taxon	Taxon voucher, herbarium, origin	GenBank accession number (Mol.)		Cyt.
		ITS (ITS1, 5.8S rRNA gene, ITS2)	*trn*T-*trn*L	
Vincetoxicum fuscatum (Hornem.) Rchb. subsp. *fuscatum* (cont.)	Güven 93 & Makbul, RUB, Niğde: Ulukışla, Maden Village, Turkey	MN106200 (PS)	MN159572 (PS)	+
	Güven 77 & Makbul, RUB, Tunceli: Pülümür, Turkey	-	-	+
Vincetoxicum glaucum (Wall. ex Wight) Rech.f.	Jan Alam 14963, KUH, Pakistan	LN880581.1 (A)	LN880805.1 (A)	
Vincetoxicum hirundinaria Medik.	Meve 970, UBT, Germany	AJ320474.1 (C)		
	Meve s.n., UBT, Germany		AJ410274.1 (D)	
Vincetoxicum hirundinaria Medik. subsp. *hirundinaria*	Güven 28 & Makbul, RUB, Kırklareli: Demirköy-İğneada road, Turkey	MN106204 (PS)	MN159576 (PS)	+
	Güven 135 & Makbul, RUB, Kırklareli: Demirköy, Mahya Mount, Turkey	MN106205 (PS)	MN159577 (PS)	+
	Güven 18 & Makbul, RUB, Kırklareli: Vize, Kömürköy, Turkey	MN106203 (PS)	MN159575 (PS)	+
Vincetoxicum intermedium Taliev	Cherkasova s.n., MW, Kazakhstan	LN880583.1 (A)	LN880807.1 (A)	
Vincetoxicum jailicola Juz.	Vylegneanina 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 nigrum (L.) Moench	New York	FJ362532.1 (E)		
	Civeyrel 1106		AY899964.1 (F)	
Vincetoxicum parviflorum Decne.	Güven 80 & Makbul, RUB, Tunceli: Ovacık, Karagöl Valley, Turkey	MN106207 (PS)	MN159579 (PS)	+
	Güven 95 & Makbul, RUB, Kayseri: Yahyalı, Kapuzbaşı Waterfalls, Turkey	MN106206 (PS)	MN159578 (PS)	
Vincetoxicum pumilum Decne.	Djavadi et al. 56807, IRAN, Iran	LN880589.1 (A)	LN880813.1 (A)	
Vincetoxicum rossicum (Kleopow) Barbar.	isolate CYKVI-4, New York	FJ517165.1 (E)		
	Murray s.n, UBT, Canada		HE793742.1 (B)	
Vincetoxicum rehmannii Boiss.	Schoenswetter et Tribsch 21,W, Georgia	LN880590.1 (A)	LN880814.1 (A)	
Vincetoxicum sakesarense Ali & Khatoon	Rizwana 036586, PMNH, Pakistan	LN880592.1 (A)	LN880816.1 (A)	
Vincetoxicum scandens Sommier & Levier	Pahlevani et Asef 27, IRAN 54575, Iran	LN880594.1 (A)	LN880818.1(A)	
	Güven 101 & Makbul, RUB, Ordu: Ünye, İnkur-Akkuş road, Turkey	MN106209 (PS)	MN159581 (PS)	

Appendix 1 (cont.)

Taxon	Taxon voucher, herbarium, origin	GenBank accession number (Mol.)		Cyt.
		ITS (ITS1, 5.8S rRNA gene, ITS2)	*trn*T-*trn*L	
Vincetoxicum scandens Sommier & Levier (cont.)	Güven 30 & Makbul, RUB, Rize: Çamlıkköy Village, Turkey	MN106208 (PS)	MN159580 (PS)	+
	Güven 121 & Makbul, RUB, Artvin: Borçka, Uğur Village, Turkey	MN106210 (PS)	MN159582 (PS)	
Vincetoxicum schmalhausenii (Kusn.) Litv.	Zernov s.n., MW, Russia	LN880595.1 (A)	LN880819.1 (A)	
Vincetoxicum speciosum Boiss. & Spruner	Güven 137 & Makbul, RUB, Kırklareli: Demirköy, Mahya Mount, Turkey	MN106214 (PS)	MN159586 (PS)	+
	Güven 43 & Makbul, RUB, Zonguldak: Çaycuma-Zongudak road, Turkey	MN106211 (PS)	MN159583 (PS)	
	Güven 112 & Makbul, RUB, Sinop: İnceburun, Turkey	MN106212 (PS)	MN159584 (PS)	
	Güven 131 & Makbul, RUB, Bursa: İnegöl, Sayfiye Village, Turkey	MN106213 (PS)	MN159585 (PS)	
Vincetoxicum stocksii Ali & Khatoon	S.I. Ali & S. Khatoon s.n., Pakistan	AJ320475.1 (C)		
	Ali & Khatoon s.n., GA, Pakistan		AJ410277.1 (D)	
Vincetoxicum tauricum Pobed.	Shvedchikova s.n., MW, Russia	LN880596.1 (A)	LN880820.1 (A)	
Vincetoxicum tmoleum Boiss.	Ehrendorfer et al. 787-84-6, W, Turkey	LN880597.1 (A)	LN880821.1 (A)	
	Güven 60 & Makbul, RUB, Ankara: Beypazarı, Karagöl Plateau, Turkey	MN106216 (PS)	MN159588 (PS)	
	Güven 123 & Makbul, RUB, Artvin: Şavşat, Meydancık Gateway, Turkey	MN106218 (PS)	MN159590 (PS)	
	Güven 48 & Makbul, RUB, Manisa: Salihli-Bozdağ road, Turkey	MN106215 (PS)	MN159587 (PS)	
	Güven 75 & Makbul, RUB, Hatay: Amanos Mountains, Turkey	MN106217 (PS)	MN159589 (PS)	
	Güven 67 & Makbul, RUB, Artvin: Artvin-Şavşat road, Turkey	–	–	+
	Güven 72 & Makbul, RUB, Erzurum: Oltu-Erzurum road, Turkey	–	–	+
Outgroup				
Cionura erecta L.	Güven 44 & Makbul, RUB, Zonguldak: Bakacakkadı, Turkey	MN106219 (PS)	MN159591 (PS)	
	Güven 54 & Makbul, RUB, Denizli: Honaz Mountain, Turkey	MN106220 (PS)	MN159592 (PS)	
	Güven 92 & Makbul, RUB, Adana: Pozantı-Ulukışla road, Turkey	MN106221 (PS)	MN159593 (PS)	
Cynanchum acutum L.	Russia	FJ572055.1 (E)		
	BG Lisbon s.n., UBT, Portugal		AJ428582.1 (G)	
Cynanchum acutum L. subsp. *acutum*	Güven 140 & Makbul, RUB, Kırklareli: İğneada, Turkey	MN106222 (PS)	MN159594 (PS)	
Gomphocarpus fruticosus (L.) W.T.Aiton	Nicholas 2796, UDW, South Africa:Eastern Cape	AM396906.1 (H)	AM295764.1 (H)	