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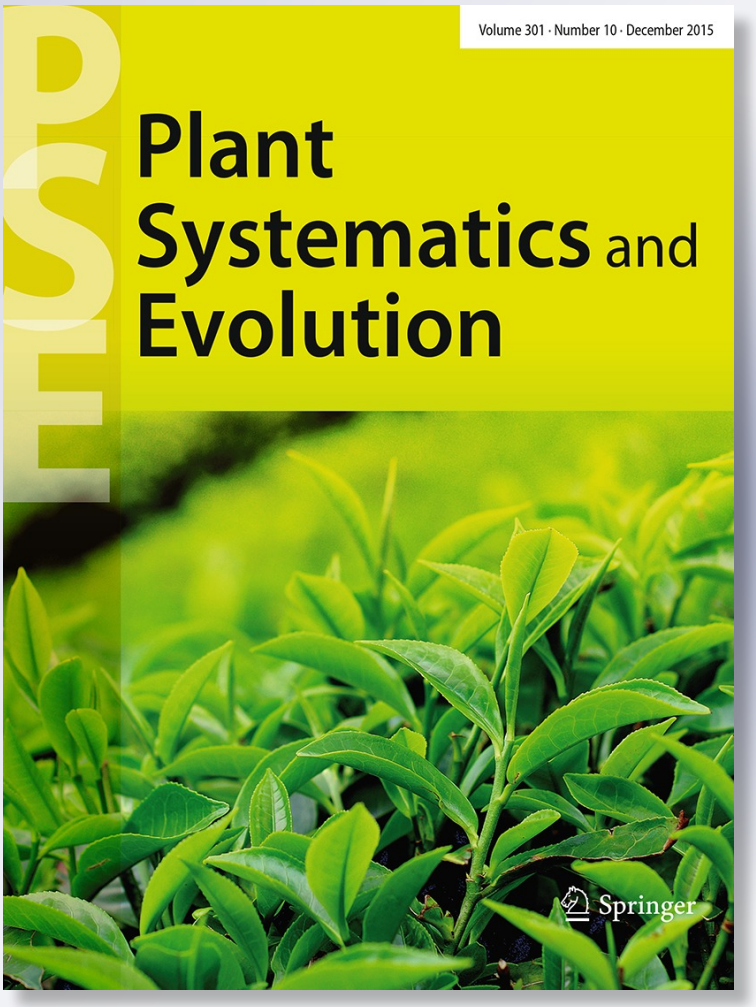

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ORIGINAL ARTICLE

A molecular phylogeny of *Salvia euphratica* sensu lato (*Salvia* L., Lamiaceae) and its closely related species with a focus on the section *Hymenosphace*

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Abstract To investigate the phylogenetic relationships of *Salvia euphratica* sensu lato and its closely related species with a focus on the section *Hymenosphace*, we screened five different regions; one nuclear ribosomal DNA region (Internal Transcribed Spacer, ITS) and four chloroplast DNA regions [trnT-trnL intergenic spacer (IGS), trnL intron, trnL-trnF IGS and trnV intron]. Based on 19 sequences of 7 *Salvia* taxa produced in the study and different number of sequences obtained from GenBank, our results supported latest taxonomic treatments on *Salvia pseudeuphratica* and *S. cerino-pruinosa* as they are resurrected and accepted different species from *S. euphratica*. The results confirmed the latest phylogenetic findings as "the section *Hymenosphace* is a non-monophyletic group, originated thick textured, non-expanding ancestral group, and expanding calyces with widely diverging lips in fruiting stage evolved several times in parallel, not only in *Salvia* but also in the Iranian genus *Zhumeria*". The species of the sect. *Hymenosphace* are mostly distributed in three different geographic regions [(1) Southwest Asia, Turkey, Russia and Iran, (2) Canary Islands, (3) Southern Africa] with different morphological characters. The results showed that ITS had the highest resolution power for discriminating studied taxa and the highest number of haplotypes was also observed in this region. The resolutions of the chloroplast regions were too low for taxa native to Turkey, but quite enough to discriminate species from the different clades whose sequences were obtained from database.

Keywords *Hymenosphace* · ITS · Lamiaceae · Molecular systematic · Phylogeny · *Salvia*

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Introduction

Salvia L. is the largest genus in the Lamiaceae, with ca. 950–1000 species, and shows high diversity in growth forms, floral morphology and pollination biology (Alziar 1988–1993; Harley et al. 2004). The genus has been widely distributed in five regions of the world; central and South America (~500 spp.), western Asia (~200 spp.), eastern Asia (~100 spp.), Africa (~60spp.) and Europe (~36 spp.) (Celep et al. 2014). Turkey is one of the centres of diversity regions in Southwest Asia with 99 *Salvia* species (Celep et al. 2014). A number of studies have been done on morphology (Hedge 1982a, b), taxonomy (Celep et al. 2009; Celep and Doğan 2010; Celep et al. 2011a, b), anatomy (Metcalfe and Chalk 1950, 1972; Kahraman et al. 2009, 2010a, b, c), micromorphology (Kahraman et al. 2011) numerical taxonomy (Reales et al. 2004), conservation biology (Celep et al. 2010), and karyology (Nakipoglu 1993; Martin et al. 2011) of the genus. However, just

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a few comprehensive molecular studies have been done on the genus until now (Walker et al. 2004; Walker and Sytsma 2007; Will and Claßen-Bockhoff 2014).

First infrageneric grouping on *Salvia* was made by Bentham (1832–1836, 1848), who described nine sections in Old World species, and then Briquet (1897) modified the Bentham's sectional delimitation. However, both infrageneric groupings are not reflecting a true picture of suprageneric level (Hedge 1974) in *Salvia*. According to Hedge (1974), current sectional delimitations are artificial and worldwide study should be done to have final conclusion on the sectional delimitation in the genus.

Salvia section *Hymenosphace* Benth. is composed of herbaceous and woody semi-shrubby plants. While the members of the section *Hymenosphace* mostly distributed in Southwest Asia, Iran, Canary Islands, Russia, Afghanistan and Southern Africa, the largest number of taxa in the section grows in Turkey (Hedge 1965, 1974) with 15 taxa, 12 of which are endemic (Kahraman et al. 2011). The characteristic feature of section *Hymenosphace* is membranous calyces which greatly enlarge after anthesis (Hedge 1974). Usually, there are four mericarps, but only one or two reach maturity (Hedge 1974; Kahraman et al. 2011). This morphological specialization could have been evolved for dispersing mericarps by wind.

Hedge (1974) recommended that section *Hymenosphace* appears to merit some kind of higher taxonomic status. Pobedimova (1954) recognized some of these species are in the independent genus *Schraderia* Medik. Then, she used the name *Arischrada* Pobed. instead of *Schraderia* (because of *Schraderia* Vahl. 1796, nom. conserv.) to cover the only Soviet species. In addition, Hedge (1974) reported that there are some transitional species between those with or without expanded calyces both in Turkey and Africa.

Salvia euphratica Montbret and Aucher ex Benth. was described in 1836 from Turkey (Bentham 1836), and then 6 more species, which are closely similar to *S. euphratica*, were described by Rechinger (1952), namely Turkish local endemics *S. leiocalycina* Rech.f., *S. cerino-pruinosa* Rech.f., *S. pseudeuphratica* Rech.f., *S. sericeo-tomentosa* Rech.f., *S. kronenburgii* Rech.f., and Iranian endemic *S. kermanshahensis* Rech.f. However, taxonomic status of those species was changed by Hedge (1982a, b). In the Flora of Turkey (Hedge 1982a), *S. leiocalycina* was treated as a variety of *S. euphratica*, on the other hand *S. pseudeuphratica* was evaluated as a synonym of *S. euphratica* var. *euphratica* and *S. cerino-pruinosa* was regarded as a synonym of *S. euphratica* Montbret and Aucher ex Benth. var. *leiocalycina* (Rech.f.) Hedge. Remaining similar species, *S. sericeo-tomentosa*, *S. kronenburgii* and *S. kermanshahensis* were conserved as distinct species. Recently, Kahraman et al. (2010a) have made extensive morphological and ecological studies on *S. euphratica* and its closely similar species to understand taxonomic relationships among the taxa. In the study of Kahraman et al. (2010a), *S. leiocalycina* accepted as a variety under *S. euphratica*, however, previously accepted synonym species of *S. euphratica*, namely *S. pseudeuphratica* and *S. cerino-pruinosa*, resurrected as distinct species. Therefore, in the present study, we referred *S. euphratica*, *S. pseudeuphratica* and *S. cerino-pruinosa* as *S. euphratica* sensu lato. In the present study, we followed taxonomic treatment of Kahraman et al. (2010a).

Molecular markers of both nuclear and chloroplast genomes are widely used to understand evolutionary relationships among taxa (Artyukova et al. 2005; Pleines et al. 2009). However, finding a suitable region for taxa is still a problem (Lahaye et al. 2008). Most of the authors indicated that internal transcribed spacer region (ITS; ITS1+5.8-S+ITS2 sub-units) of 18S–26S nuclear ribosomal DNA (nrDNA) has frequently been preferred for molecular studies (Baldwin 1992; Wojciechowski et al. 1993; Wojciechowski 2005; Yao et al. 2010). Variation levels of this region are suitable for phylogenetic inference at the specific, generic or even family levels (Baldwin 1992; Baldwin et al. 1995). In addition to nuclear genome, several chloroplast DNA regions are also widely used for phylogenetic studies (Shaw et al. 2005). In the present study, DNA sequences of four chloroplast DNA regions [trnT-trnL intergenic spacer (IGS), trnL intron, trnL-trnF IGS and trnV intron] were used.

Recent molecular studies (Walker et al. 2004; Walker and Sytsma 2007; Will and Claßen-Bockhoff 2014) have shown that *Salvia* is non-monophyletic. In their genus-wide study, Walker and Sytsma (2007) proposed three major clades in *Salvia*. Then, Will and Claßen-Bockhoff (2014) modified Walker and Sytsma's clades and accepted four major clades as "Clade III is paraphyletic with respect to *Zhumeria majdae* Rech.f. and Wendelbo and the East Asian species constituting the fourth independent evolutionary lineage as Clade IV". In this study, we adopted phylogenetic approach, clade names and stamen types of Will and Claßen-Bockhoff (2014).

Nine species of the sect. *Hymenosphace* from Iran, Southern Africa and Canary Islands and *Zhumeria majdae* from Iran were included in the study of Will and Claßen-Bockhoff (2014). In their study, six Southern African species, *Salvia dolomitica* Codd., *S. chamelaeagnea* P.J. Bergius, *S. lanceolata* Lam. and *S. albicaulis* Benth., *S. lutea* L. (=*S. africana-lutea* L.) and *S. africana* L. (=*S. africana-caerulea* L.), were placed in the sub-clade IA, *Salvia canariensis* L. from Canary Island was placed in sub-clade IC, *Salvia garipensis* E.Mey. ex Benth. from Southern Africa and *S. hydrangea* DC. ex Benth. from Turkey, Russia and Iran was placed in sub-clade ID, and *Zhumeria majdae* was placed in clade III. However,

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Turkish local endemic species, which were used in this study, *Salvia euphratica* var. *euphratica*, *Salvia euphratica* var. *leiocalycina*, *S. pseudeuphratica*, *S. cerino-pruinosa*, *S. kronenburgii*, *S. sericeo-tomentosa* Rech.f. var. *sericeo-tomentosa* and *S. sericeo-tomentosa* Rech.f. var. *hatayica* Celep and Doğan have not been included in any phylogenetic studies (Walker et al. 2004; Walker and Sytsma 2007; Zhang et al. 2008; Xu et al. 2009; Takano and Okada 2011; Wang et al. 2013; Will and Claßen-Bockhoff 2014) until now.

The main objectives of the study were to test recent taxonomic treatments of *S. euphratica* sensu lato and its closely similar species made by Kahraman et al. (2010a) based on DNA sequences of both nuclear and chloroplast DNA regions (additional new 7 taxa, including 19 new sequences from one nrDNA and 4 chloroplast markers for *Salvia* phylogeny), to clarify shared characters of different origin (parallel or convergent characters) and phytogeography of the section *Hymenosphace* with increased sample size, and to add new data on *Salvia* phylogeny.

Materials and methods

Plant materials

Plant specimens were collected from their natural habitats in the different parts of Turkey. Plants were identified by Dr. F. Celep and Dr. A. Kahraman according to the diagnostic morphological characteristics described in the Flora of Turkey and the East Aegean Islands (Hedge 1982a) and voucher specimens deposited Department of Biological Sciences, Middle East Technical University (METU), Ankara. Habit and flower photos of the studied taxa are given in Fig. 1. For each taxon, 2 or 3 accessions were collected (Table 1) and preserved in plastic bags with silica gel until DNA extraction. To increase the interspecific sampling, sequences from earlier investigations were obtained from GenBank (Online Resource 1). *Rosmarinus officinalis* L. (KJ584197), *Isodon umbrosus* (Maxim.) H.Hara (AB523500), *Lamium purpureum* L. (JF780009), and *Origanum vulgare* L. (JX880022) were selected as

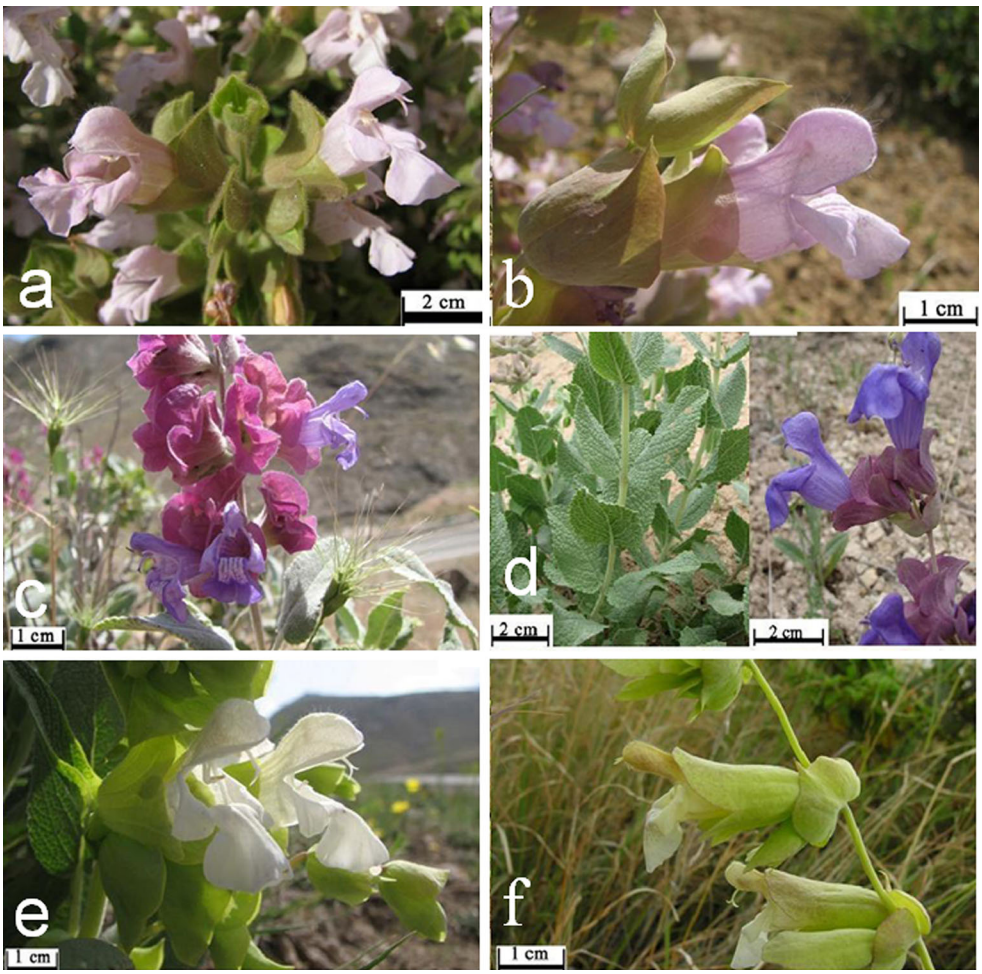

Fig. 1 Calyx and corolla of the studied taxa. **a** *Salvia euphratica* var. *euphratica*, **b** *S. euphratica* var. *leiocalycina*, **c** *S. pseudeuphratica*, **d** *S. cerino-pruinosa*, **e** *S. kronenburgii*, **f** *S. sericeo-tomentosa*. (Photos modified from Kahraman et al. 2010a)

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Table 1 Samples of *Salvia* taxa used in the study

Taxon	Locality[a]	# of Sample	Accession number[b] (ITS)	Accession number[b] (trnT-L IGS)	Accession number[b] (trnL intron +trnL-F IGS)	Accession number[b] (trnV intron)
S. euphratica var. *euphratica*	Sivas/Malatya	3	KM519756	KM519763	KM519770	KM519777
S. euphratica var. *leiocalycina*	Sivas/Malatya	3	KM519757	KM519764	KM519771	KM519778
S. cerino-pruinosa	Sivas	3	KM519758	KM519765	KM519772	KM519779
S. kronenburgii	Van	3	KM519759	KM519766	KM519773	KM519780
S. sericeo-tomentosa var. *sericeo-tomentosa*	Hatay	2	KM519760	KM519767	KM519774	KM519781
S. sericeo-tomentosa var. *hatayica*	Hatay	2	KM519761	KM519768	KM519775	KM519782
S. pseudeuphratica	Elazıg	3	KM519762	KM519769	KM519776	KM519783

Location, number of sample and accession number for each used region

[a] Each word after '/' indicates different location. If all specimens of a species were collected from same location '/' was not used

[b] DNA sequences of 2 or 3 samples of one taxa were identical. Therefore, only one of them was submitted to the GenBank

Table 2 PCR reaction conditions for each region

PCR reaction condition	Regions				
	nrDNA	cpDNA			
	ITS (μl)	trnT-L IGS (μl)	trnL intron (μl)	trnL-F IGS[a]	trnV intron[a]
DNA (10 ng/μl)	2	3	2		
10X PCR Buffer	3	3	2		
$MgCl_2$	2	3	2		
dNTP mixture	2	2.5	2		
Primer pair	1 + 1	2 + 2	1 + 1		
Taq polymerase	0.2	0.3	0.2		
H_2O	38.8	34.2	39.8		

[a] Volumes used to amplify the region are same with those of trnL intron

outgroup for the phylogenetic analysis of ITS, trnT-trnL IGS, trnL-trnF, and trnV intron regions, respectively.

DNA isolation, PCR amplification, and sequencing

Total genomic DNA was extracted from dried or fresh leaf tissues using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). The purity and quantity of genomic DNA were determined by NanoDrop 2000c UV–Vis Spectrophotometer (Thermo Scientific) and using 1.0 % agarose gel electrophoresis. After isolation, samples were stored at −20 °C prior to amplification. To determine the comparative performance of different DNA markers, each sample was analysed with different candidate regions. These included four regions (trnT-trnL IGS, trnL intron, trnL-trnF and trnV intron) in chloroplast and one region (ITS) in nuclear genome. The DNA sequences of primers for each region were obtained from previous studies (Taberlet et al. 1991; Wang et al. 1999; Hsiao et al. 1995). DNA amplification was performed in a 50 μl volume containing genomic DNA (10 ng/μl), 10X PCR Buffer [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1 % Tween 20], $MgCl_2$ (25 mM), dNTP mixture (10 mM), selected primer pair (10 μM), Taq polymerase (5u/μl) and sterile water. Volumes of them were decided after optimization studies (Table 2). The reaction mixtures were amplified in a DNA Thermal Cycler (Eppendorf Mastercycler 5333 version 2.30.33-09). PCR amplification was always started with 10 min initial denaturation at 95 °C, and terminated with 5 min at 72 °C. Number of cycles, temperature and length of time for denaturation, primer annealing and extension steps are given in Table 3. Amplicons were visualized by electrophoresis on 1–1.5 % agarose gels. Purified PCR products were sequenced in both directions using ABI 310 Genetic Analyzer (PE Applied Biosystem) Automatic Sequencer (RefGen Biotechnology, Ankara).

Phylogenetic analyses

The nucleotide sequences of each region were aligned with ClustalW program (Thompson et al. 1994) using the following parameters: pairwise alignment gap opening = 15,

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Table 3 The thermal cycling parameters for each region

Region	Step	Temperature (°C)	Time	Cycle #	Description
ITS	1	95	10′	1	Denaturation
	2	94	45″	30	Denaturation
		50	30″		Annealing
		72	45″		Extension
	3	72	5′	1	Final extension
trnT-L IGS	1	95	10′		Denaturation
trnL intron	2	94	30″	30	Denaturation
trnL-F IGS		53 (58)	25 (30)″		Annealing
(trnV intron)		72	30 (35)″		Extension
	3	72	5′	1	Final extension

gap extension = 6.6 and multiple alignment gap opening = 15, gap extension = 6.7, delay divergent sequences = 30 % and transition weight = 0.5. Alignments were checked and manually adjusted where it is necessary. All sequences were combined with sequences of used regions from GenBank and analysed together. Alignments used for phylogenies are available in Online Resource 2.

During the analyses, indels were coded as missing values and the sites containing missing data or alignment gaps were removed from the analyses using pairwise-deletion option in Molecular Evolutionary Genetics Analysis software (MEGA 5.0; Tamura et al. 2011). Phylogenetic trees were constructed using maximum likelihood (ML) method based on the Tamura–Nei model (1993) and support for branches was estimated using bootstrap analysis with 500 replications (Felsenstein 1985).

Phylogenetic trees sometimes may not completely measure gene genealogies of haplotypes resulting from intraspecific polymorphisms (Clement et al. 2000; Posada and Crandall 2001). Therefore, in the current study, parsimony network analysis (TCS) was also used to obtain more accurate relationships among ingroup haplotypes plus three closest species as outgroup. Haplotype cladogram was constructed using the program TCS 1.21 (Templeton et al. 1992; Clement et al. 2000). The software estimates the maximum number of differences among haplotypes as a result of single substitutions with a 95 % statistical confidence (parsimony connection limit). Furthermore, lowered connection limit (94 %) was also used to see relationships among the distantly related haplotypes. The most informative region (ITS nrDNA) was selected to show haplotypes. All sites were weighted equally and the gap was treated as 5th state during the procedure.

Results

DNA sequences of taxa used in current study (Online Resource 2) were assembled with those of species retrieved from GenBank and analysed together in MEGA 5 software. The final ITS data set composed of sequences of 19 accessions of 7 *Salvia* taxa and 58 *Salvia* accessions from GenBank. Total length of the region of the species used in the current study was 629 bp except *S. sericeo-tomentosa* var. *sericeo-tomentosa* and *S. sericeo-tomentosa* var. *hatayica* (628 bp). However, the total aligned ITS sequences (with species obtained from GenBank) yielded 648 characters; 184 of which were parsimony informative. There was no genetic variation among sequences of repeated samples of each taxon. Even though only one indel and 18 substitutions were observed in the aligned data of Turkish species, lots of indels with variable lengths and more than 258 variation sites were detected in the ITS data when sequences from GenBank were added.

The data set of trnT-trnL IGS region composed of 670 characters with one indel and one nucleotide substitution that were observed at positions 245 and 344, respectively. This genetic variation was detected in the sequence of *S. sericeo-tomentosa* var. *sericeo-tomentosa* and *S. sericeo-tomentosa* var. *hatayica* and caused phylogenetic separation from others. Unfortunately, only one sequence of the trnT-trnL IGS region was found in GenBank (*S. miltiorrhiza* Bunge) and when it was included in the analysis, the length of the region was calculated as 725 bp because of several insertions. In the combined data, 34 variable sites were detected but only one of them was parsimony informative. The aligned sequences of trnL intron region yielded total lengths of 461 nucleotides, and no substitution was observed among sequences when only native taxa were analysed. Same situation was also observed in the sequence of trnL-trnF region (382 bp; including tRNA-Leu complete and tRNA-Phe partial DNA sequences). Therefore, DNA sequences of two regions were combined and analysed with the total sequences of trnL-trnF (trnL intron+trnL-trnF IGS) region of 12 species which were retrieved from GenBank. The total length of the region was calculated as 843 bp for Turkish species. When species from GenBank were not included in the analysis, only two indels were observed and no substitution was there. The other species

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whose sequences were taken from GenBank had too many indels and substitutions in the aligned data and caused the region to extend (913 bp).

Finally, DNA sequence of trnV intron region was used to understand suitability of the region for phylogenetic studies of *Salvia*. The total data set of 20 sequences from 8 taxa contained 558 characters, and only 1 of those were parsimony informative. As like trnT-L IGS region, only one DNA sequence of the region was retrieved from GenBank and analysed with our data set. No indel was detected when only sequences of native species were analysed and 2 indels were seen when sequence of *Salvia miltiorrhiza* from GenBank was added.

Evolutionary divergence

Evolutionary divergence between taxa was conducted using the Maximum Composite Likelihood model (Tamura et al. 2004, MEGA 5). No intraspecific divergence was detected among accessions of one taxon so they were grouped together without any distance in '*Salvia* phylogenetic trees' (Fig. 2 and Online Resource 1—Figs. S1–S3). Among studied regions, sequence of ITS showed the highest number of nucleotide substitution. This divergence caused separation of each species in the dendrogram (Fig. 2). *Salvia euphratica* has 2 varieties named as *S. euphratica* var. *leiocalycina* and *S. euphratica* var. *euphratica.* However, no genetic variation was observed among accessions of these varieties and accessions grouped in a sub-cluster when ITS and trnV regions were used to construct the trees (Fig. 2 and S3). *Salvia pseudeuphratica* was previously treated as a synonym of *S. euphratica* var. *euphratica* in the Flora of Turkey (Hedge, 1982a). However, these two species evolutionary separated in the trees constructed by ITS and trnV regions (Fig. 2 and S3). Moreover, in the Flora of Turkey, *S. cerino-pruinosa* was expressed as a synonym of *S. euphratica* var. *leiocalycina,* however samples of these species phylogenetically separated and caused two different sub-clusters in the ITS and trnV trees (Fig. 2 and S3). Therefore, our phylogenetic results supported recent taxonomic treatments of *S. euphratica* sensu lato and its closely similar species made by Kahraman et al. (2010a). In addition to the constructed phylogenetic trees based on each used region (Fig. 2 and Online Resource 1—Figs. S1–S3), one more tree was constructed using combined data to find out questionable or unresolved parts of the former trees. Combined-data tree indicated fairly same structure of the ITS tree (Fig. 2) so combined-data tree was not given as a different figure.

Few or no substitution was observed in the sequences of regions found in chloroplast DNA (trnT-trnL IGS, trnL-trnF and trnV). Samples of *S. sericeo-tomentosa* (var. *sericeo-tomentosa* and var. *hatayica*) were clustered together when ITS and trnT-L IGS regions were analysed separately. In the trnT-trnL tree, all taxa except *S. sericeo-tomentosa* grouped together since no genetic divergence was detected among taxa except *S. sericeo-tomentosa* (Online Resource 1—Fig. S1). trnL-trnF phylogenetic tree had a simple pattern having all studied species grouped together and separated from most of the species retrieved from GenBank (Online Resource 1—Fig. S2). Individuals of *Salvia euphratica* (var. *euphratica* and var. *leiocalycina*) phylogenetically separated from others and caused a cluster in the trnV intron tree (Online Resource 1—Fig. S3).

On the basis of the ITS sequences, parsimony network analysis generated eight haplotypes when gaps were considered as 5th state (Fig. 3). Five (H1, H2, H3, H4, H5) discrete sequence groups within ingroup were identified and each mutation between haplotypes was indicated by black (95 %) or open (94 %) nodes on the branches. Three outgroup species (H6, H7, H8) were unconnected to ingroup haplotypes even though 94 % connection limit was used (Fig. 3). Rectangular shaped haplotype indicates the ancestral, while oval one shows derived haplotypes. The statistical parsimony network showed the close relationships among the ingroup species except *S. sericeo-tomentosa* var. *sericeo-tomentosa* and *S. sericeo-tomentosa* var. *hatayica* (H5, 94 % connection limit). Haplotype 1 (H1, *S. euphratica*) is most likely ancestral species for ingroup taxa (Fig. 3). *Salvia euphratica* (H1) was closely related to *S. cerino-pruinosa* (H2) and *S. pseudeuphratica* (H3), which were only two and one mutations apart from H1 haplotype, respectively. Haplotype network analysis delimited the taxa which is congruent with the ITS phylogenetic tree (Fig. 2).

Will and Claßen-Bockhoff (2014) reported that taxa of the section *Hymenosphace* were grouped in different subclades of large Clade I. For example, Southwest Asian, Turkish and Iranian taxa were placed in the sub-clade ID, Southern African taxa were placed in mostly sub-clade IA and *Salvia canariensis* from Canary Islands was placed in sub-clade IC. One interesting result is that Southern African species *S. garipensis* in sect. *Hymenosphace* was placed in sub-clade ID together with Southwest Asian, Turkish and Iranian taxa rather than in South African sub-Clade IA. On the other hand, *Zhumeria majdae* having expanding fruiting calyx was placed in sub-clade IIIB with Iranian and Turkish species *S. aristata* Aucher ex Benth which has no expanding fruiting calyx. East Asian taxa were clearly separated from the other taxa and placed in Clade IV (Fig. 2). Our results are completely congruent with phylogenetic findings of Will and Claßen-Bockhoff (2014). Using the same clade names from Will and Claßen-Bockhoff (2014), our studied Turkish taxa in this study were placed in the sub-Clade ID (Fig. 2).

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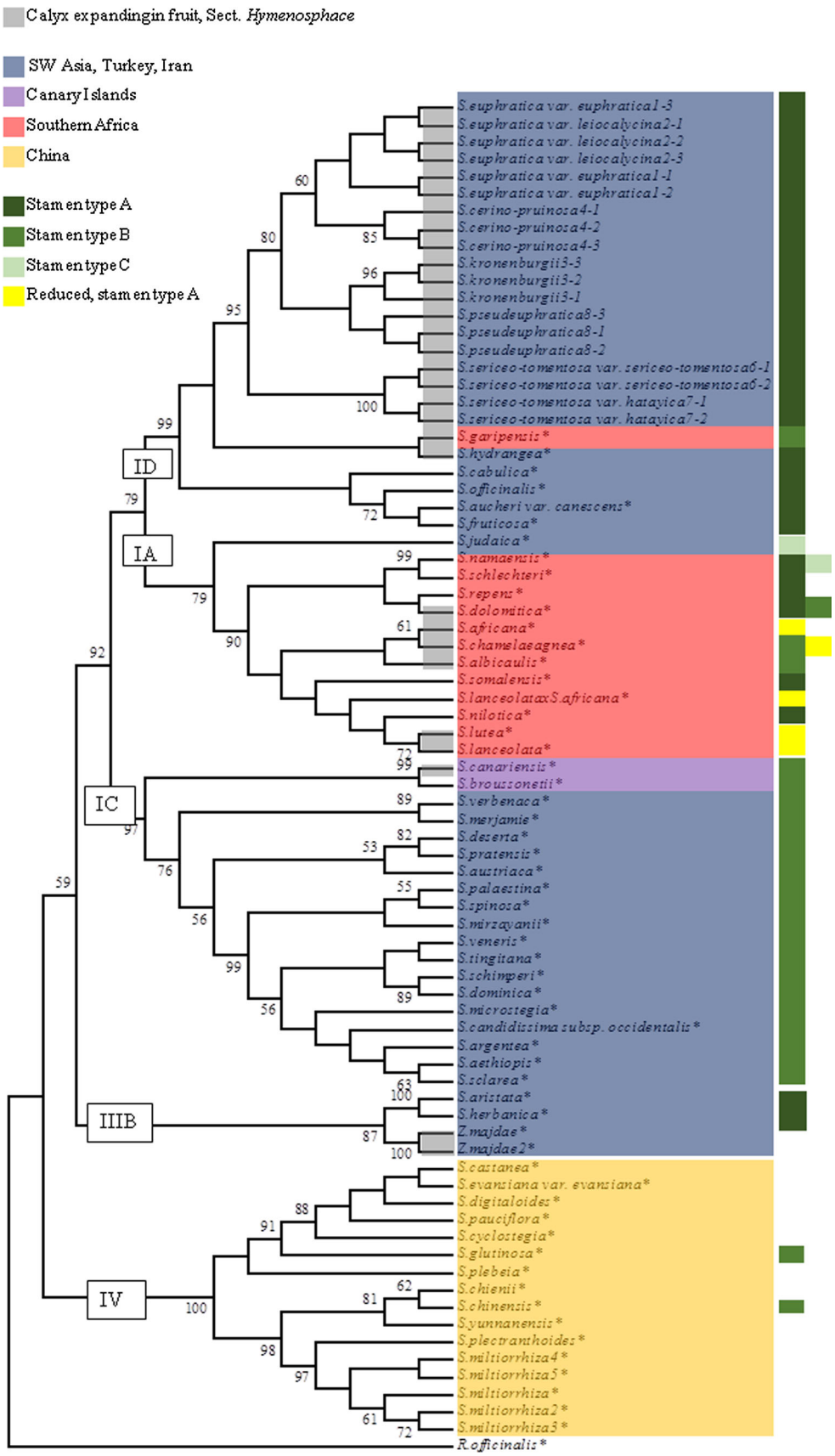

Fig. 2 Phylogeny of *Salvia* taxa based on DNA sequence of nrDNA ITS region and combined data. *Bootstrap values* are indicated above or below of appropriate nodes for which support values were greater than 50 %. (*Asterisk*: species retrieved from GenBank). (For stamen types and clade names, see Will and Claßen-Bockhoff 2014). DNA sequence of the two species, *S. lutea* and *S. africana,* was taken from GenBank, however, according to current taxonomic treatment, *Salvia lutea* is a synonym of *S. africana-lutea* L. and *S. africana* is a synonym of *S. africana-caerulea* L

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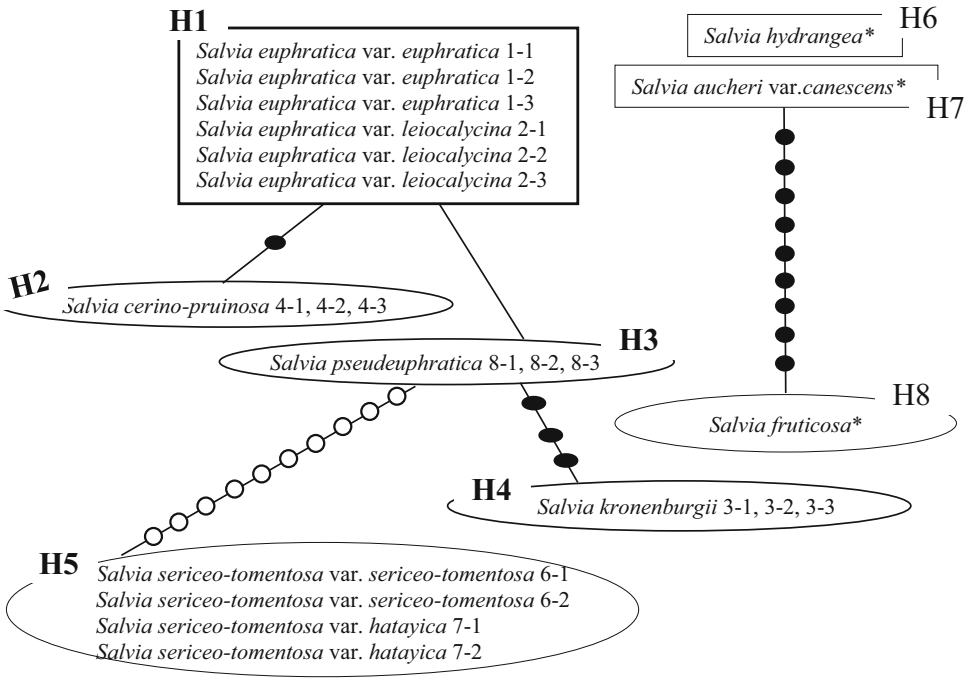

Fig. 3 Haplotype networks for nrDNA ITS region under the 95 % (94 % to see networks of distantly related haplotypes) parsimony criterion. More than one name in a frame indicates identical genotypes. The rectangle framed with *thicker line* (H1) represents the haplotype most likely to be ancestral for native *Salvia* species. The number of steps (synonymous with intermediate or unsampled haplotypes) separating different haplotypes is represented by *black nodes* (95 % connection limit) and *open nodes* (94 % connection limit) appearing on the branches. Each node represents a single mutation event and the branch length is meaningless. *Asterisk* species retrieved from GenBank

In sub-clade ID (Fig. 2), all taxa of the sect. *Hymenosphace* from Southwest Asia, Turkey and Iran have stamen type A (see Fig. 8 of Will and Claßen-Bockhoff 2014). Only South African species, *S. garipensis* has stamen type B. In addition, there are four sister species (*S. officinalis* L., *S. fruticosa* Mill., *S. aucheri* Benth.var. *canescens* (Boiss & Heldr.) and *S. cabulica* Benth.) which have not expanding fruiting calyx in sub-clade ID. These four species were traditionally placed in the sect. *Salvia* (=*Eusphace* Benth.) by Hedge (1965, 1972) and Celep (2011b). Sub-clade IA mostly includes Southern African species from the section *Hymenosphace* and the other sections (i.e. *S. schlechteri* Briq., *S. repens* Burch. ex Benth., *S. somalensis* Vatke, *S. nilotica* Juss. ex Jacq. and *S. namaensis* Schinz). In Southern African species of sect. *Hymenosphace*, *S. dolomitica* has both stamen type A and B, *S. lutea*, *S. lanceolata*, *S. lanceolata x S. africana* (=*S. lanceolata x S. africana-caerulea*) and *S. africana* have reduced stamen type A, *S. albicaulis* has stamen type B, and *S. chamelaeagnea* has both reduced stamen type A and stamen type B. The other species, which were not placed in the sect. *Hymenosphace* in sub-clade IA, *S. schlechteri*, *S. repens*, *S. somalensis* and *S. nilotica,* have stamen type A and *S. namaensis* has both stamen type B and C (Fig. 2). Two major lineages are recognized in sub-clade IC. One lineage contains the two Canary Island endemic species *S. broussonetii* Benth. and *S. canariensis*. While the former species has thick textured calyx, the latter one has expanding fruiting calyx and therefore placed in the sect. *Hymenosphace*. Another lineage contains some European and Southwest Asian species (see Fig. 2) from the different sections, i.e. *S. verbenaca* L. (sect. *Plethiosphace*), *S. sclarea* L. (sect. *Aethiopis*) and *S. aethiopis* L. (sect. *Aethiopis*). All species in sub-clade IC have stamen type B. Iranian/Turkish species *Salvia aristata* and Iranian monotypic genus, *Zhumeria majdae,* were placed in the sub-clade IIIB, the latter species has expanding fruiting calyx. *Salvia aristata* has recently been reported from Turkey (Behçet and Avlamaz 2009). Well-supported Clade IV has mostly East Asian species and one widely distributed species, *S. glutinosa* L., known from Europe, Turkey, Russia and China.

In the phylogenetic trees (Fig. 2 and Online Resource 1—Figs. S1–S3), species obtained from GenBank were generally separated from the species used in the present study. Separation of species obtained from GenBank is meaningful since they are not native to Turkey and high genetic variation was observed. In the ITS (Fig. 2) and trnL-trnF (Online Resouce 1—Fig. S2) trees, abroad species (GenBank) grouped according to their origins. Same situation was observed in trnL-trnF tree; species native to America and China grouped separately.

Discussion

Kahraman et al. (2010a) based on morphometric multivariate analysis on *S. euphratica* sensu lato and its closely related species resurrected two species *Salvia pseudeuphratica* and *S. cerino-pruinosa*, since previously both species were treated as synonyms under *Salvia euphratica*

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var. *euphratica* and *S. euphratica* var. *leiocalycina*, respectively, by Hedge (1982a). Kahraman et al. (2010a) reported that though *S. euphratica* sensu lato and its closely related species have shruby woody stems at least below, clearly expanding calyces in fruiting stage up to 30–45 mm, and elliptic to ovate–oblong leaves, there are clear morphological differences among them. For example, *S. pseudeuphratica* clearly differs from *S. euphratica* on its densely white lanate indumentum, shorter and erect stems, denser indumentum in fruiting stage, smaller, greyish and non-membranous bracts, smaller and entirely purplish calyces and smaller corollas and nutles. Similarly, *S. cerino-pruinosa* clearly differs from *S. euphratica* by having erect and mainly glabrous–pruinose indumentum, oblong–lanceolate to elliptic leaves with always one pair of very small lateral lobes, very short petiole, and sessile stem leaves. Our molecular phylogenetic results confirm taxonomic findings of Kahraman et al. (2010a). Accessions of *S. euphratica* var. *leiocalycina* and var. *euphratica* grouped distinctly from *S. pseudeuphratica* and *S. cerino-pruinosa* in the trees constructed by ITS and trnV regions (Fig. 2, Online Resource 1—Fig. S3). However, no genetic variation between varieties of *S. euphratica* (var. *leiocalycina* and var. *euphratica*) was observed so they could not be separated using molecular techniques even though Kahraman et al. (2010a) separated them using morphological characters such as indumentum, inflorescence, bract, and calyx structures. It can be concluded that none of the used regions are suitable to separate varieties of *Salvia* species.

Ninety-four percent parsimony criterion revealed five haplotypes of seven ingroup taxa in a single network (Fig. 3), thus affirming that all taxa closely related to one another. H1 haplotype containing sequences of *S. euphratica* var. *euphratica* (H1) and *S. euphratica* var. *leiocalycina* (H1) was considered as ancestral species. Only *S. sericeo-tomentosa* var. *sericeo-tomentosa* (H5) and *S. sericeo-tomentosa* var. *hatayica* (H5) seem to be distant from the other ingroup haplotypes in the network. This may be due to its morphologic, ecologic and phytogeographic differences from the other ingroup haplotypes. *Salvia sericeo-tomentosa* (H5) clearly differs from the other haplotypes on its clearly attenuate, narrowly oblong, and sericeo-tomentose leaves, white or cream corollas with yellow upper lips, and always branched inflorescence. It grows on calcareous slopes and open *Pinus* forests, roadsides with *Quercus* scrubs between 20 and 1000 m. Among the studied taxa, only *S. sericeo-tomentosa* grows in the Mediterranean phytogeographic region, the other studied taxa grow in the Irano-Turanian phytogeographic region. Parsimony network analysis further reinforced the distinct status of outgroup taxa (*S. aucheri* var. *canescens, S. fruticosa, S. hydrangea*) by excluding them from the main network (Fig. 3).

Our results are highly congruent with Will and Claßen-Bockhoff's findings (2014) as "the sect. *Hymenosphace* in the genus *Salvia* is a non-monophyletic group" and "expanding calyces with widely diverging lips in fruiting stage evolved several times in parallel, not only in *Salvia* but also in *Zhumeria*". Constructed phylogenetic ITS tree of the present study and Will and Claßen-Bockhoff (2014) indicated that taxa of the sect. *Hymenosphace* in Old World are distributed in three different regions as first region is Southwest Asia, Turkey, Iran, Afghanistan and Russia, the second region is Canary Islands and the third one is Southern Africa. Taxa of the sect. *Hymenosphace* from the different geographical regions have different morphology. For example, Southwest Asian, Turkish and Iranian taxa have clearly straight upper lip of corolla, on the other hand the taxa from Canary Islands and Southern Africa have slightly or clearly falcate upper lip. *Salvia canariensis* differs from the other taxa in the sect. *Hymenosphace* by its sagitate to hastate leaf bases (Hedge 1974). Recently, two phylogenetic studies on the genus (Walker et al. 2004; Will and Claßen-Bockhoff 2014) have been proved non-monophyly of the genus. This is also true for the sect. *Hymenosphace* in the genus *Salvia*. Our and Will and Claßen-Bockhoff's (2014) results showed that though all taxa from the sect. *Hymenosphace* were placed in large Clade I, they are placed in different sub-clades, i.e. sub-clade IA, IC and ID. All Turkish taxa from the sect. *Hymenosphace* were placed in sub-clade ID with some other species from the sect. *Salvia* which differs from the sect. *Hymenosphace* by its non-membranous and not or scarcely expanding calyces in fruiting stage. Similar case is true for Southern African species from the sect. *Hymenosphace*, all of them placed in sub-clade IA with other species out of the sect. *Hymenosphace*. From Southern Africa, *S. garipensis* from the sect. *Hymenosphace* was only placed in sub-clade ID. *Salvia canariensis* (in sect. *Hymenosphace*) from Canary Islands was placed in sub-clade IC with the other Canary Island endemic species, *S. broussonetii* (in sect. *Aethiopis*, Hedge, 1974), and some additional species from Southwest Asia, Turkey and Europe (i.e. *S. verbenaca* from the sect. *Plethiosphace*, *S. aethiopis*, and *S. sclarea* from the sect. *Aethiopis*, see Fig. 2). These geographically isolated groups in different clades seem to be monophyletic. In our current knowledge, it is not necessary to apply a higher taxonomic level on the sect. *Hymenosphace* as Hedge (1974) stated. The studies on the sect. *Hymenosphace* should be expanded with additional specimens and species from its whole distribution range, particularly from Turkey and Africa where some transitional species occur, i.e. *S. absconditiflora* Greuter & Burdet, *S. multicaulis* Vahl, *S. cadmica* Boiss., *S. blepharochlaena* Hedge & Hub.-Mor., *S. smyrnaea* Boiss. (transitional form), and *S. anatolica* Hamzaoğlu & A.Duran (transitional form).

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Specifying a suitable region for phylogenetic studies is very challenging process. A region carrying high interspecific divergence would be useful for phylogenetic studies because this region may distinguish different species in the constructed phylogenetic tree (Gao et al. 2010). The results showed that *Salvia* taxa native to Turkey could be clearly distinguished from the species obtained from GenBank whichever region was analysed. However, parameters of genetic divergence, parsimony network analysis and constructed phylogenetic trees indicated that nuclear ITS region had an advantage compared to regions located in chloroplast DNA. Evolutionary tree constructed based on sequence of ITS region showed the highest interspecific divergence so it provided much better resolution of relationships among *Salvia* taxa (Fig. 2). The study highlights that the most potential region should be found before starting a phylogenetic study. Although our sampling was limited to a single genus, it was quite enough to clarify which region would be useful for further studies. Our study shows that (1) ITS in the nuclear genome is the best region or marker among the considered regions with sufficient variability and high discrimination efficiency. Moreover, lots of representative sequences are there in database which would be valuable to understand evolutionary relationships of *Salvia* species in the world. (2) The TCS analysis of ITS region produced five connected haplotype networks (94 % connection limit) among the ingroup taxa and *S. euphratica* were considered as ancestral for ingroup haplotypes. (3) Within studied chloroplast regions, trnL-trnF showed higher resolution in the dendrogram even if there was lack of resolution when only taxa native to Turkey were analysed. (4) Representative DNA sequences of trnV and trnT-L IGS regions are not found in the reference database; so more new sequences and/or further studies are needed to indicate usefulness of these regions for studying phylogeny of *Salvia*.

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Conflict of interest The authors declare no conflict of interest.

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