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Stachys vuralii (Lamiaceae), a new species from north Anatolia, Turkey

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Stachys vuralii Yıldız, Dirmenci & Akçiçek (Lamiaceae), a new species of the section *Eriostomum* is described from north Anatolia, Turkey. Detailed illustrations and taxonomic comments are provided along with a table listing the differential characters to the closely related *S. byzantina* and *S. thirkei*. Delimitations towards *S. byzantina* and *S. thirkei* and existence of putative hybrids are discussed. A phylogenetic analysis using ITS of nuclear ribosomal DNA confirmed the status of *S. vuralii* as a distinct species. The geographical location of *S. vuralii* is also presented.

With ca. 300 taxa, *Stachys* is among the largest genera of Lamiaceae. This cosmopolitan genus is centered in the warm temperate regions of the Mediterranean and SW Asia, with secondary centers in North and South America and southern Africa. The majority of the species grow in rocky places, mainly on limestone and other basic rocks (Bhattacharjee 1974, 1980, Harley *et al*. 2004).

In Turkey, 82 species (107 taxa) are found representing two subgenera and 15 sections. Fifty-one (48%) of these taxa are endemic to Turkey, mostly constituting part of the East Mediterranean floristic elements. After the generic revision by Bhattacharjee (1982), 12 new taxa have been described from Turkey (Bhattacharjee 1974, 1980, Davis *et al*. 1988, Yıldız & Tan 1988, Gemici & Leblebici 1988, Sümbül 1990, Duman 2000, Dinç & Doğan 2006, İlçim *et al*. 2008, Daşkın *et al*. 2009, Akçiçek 2010).

Although various features, including pollen morphology, can be used for differentiating some *Stachys* taxa (Salmaki *et al*. 2008), utilization of ITS phylogeny in plants has been effective (Baldwin *et al*. 1995, Álvarez & Wendel 2003) and it has been used for Lamiaceae in multiple reports (e.g. Steane *et al*. 1999, Prather *et al*. 2002, Bräuchler *et al*. 2010, Dirmenci *et al*. 2010). In this study, we utilized ITS phylogeny along with morphological and karyological analyses to assess the taxonomic position of an unidentified specimen. Combined results suggested it was a new species in the genus *Stachys*, sect. *Eriostomum*. A comprehensive revision involv-

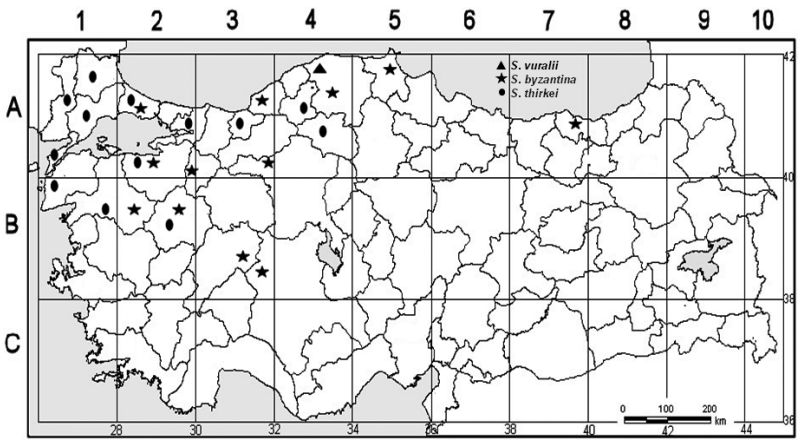

Fig. 1. Geographical distribution of *Stachys vuralii*, *S. byzantina* and *S. thirkei* in Turkey.

ing morphology and ITS phylogeny of *Stachys* sect. *Eriostomum* completed by us (unpubl. data) further confirmed these results.

Material and methods

Specimen collection

During an expedition to north Anatolia in the context of a revisionary study of *Stachys* sect. *Eriostomum* in August 2007, some unusual specimens of *Stachys* were collected in the Bartın province (Fig. 1). The specimens were examined using relevant literature (Koch 1848, Ball 1968, Knorring 1977, Bhattacharjee 1982, Rechinger 1982, Davis *et al*. 1988, Baden 1991, Duman 2000). Extensive herbarium studies were performed on relevant specimens collected previously from Turkey and the adjacent countries in addition to specimens housed in the herbaria ANK, AEF, BM, E, EGE, G, HUB, ISTE, ISTF, K, W, and WU. As a result, the Bartın specimens were confirmed to represent a new species with morphological affinities to *S. byzantina* and *S. thirkei*.

Chromosome analysis

Cytological observations on *S. vuralii*, *S. byzantina* and *S. thirkei* were made on mitotic metaphase cells of root tips obtained from germinating seeds. Root tips were pre-treated for 16 h in α-monobromonaphthalene at 4 °C and washed and fixed in Carnoy solution (3:1 absolute ethanol:glacial acetic acid) overnight. The root tips were hydrolyzed for 10 min in 1 N HCl at room temperature, washed with distilled water and stained in 2% aceto-orcein for 2 h. Stained root tips were then squashed in a drop of 45% acetic acid and permanent slides were made by mounting in Depex.

Genomic DNA isolation, PCR and sequencing

Total genomic DNA isolation was performed using Plant DNeasy kit (Qiagen GmbH, Hilden, Germany). PCR was run using the published ITS primers (White *et al*. 1990, Sang *et al*. 1995) with the following protocol in a Thermo Px2 Thermal Cycler (Thermo, U.S.A.): 5 min 95 °C initial denaturation, 35 cycles of 30 s 94 °C denaturation, 30 s 50 °C annealing and 1 min 72 °C extension, followed by a 10 min final extension at 72 °C. The primers used to amplify the ITS regions were also used for sequencing at RefGen Inc. (Ankara, Turkey) using an ABI 3130XL Genetic Anaylzer (Applied Biosystems, Fostercity, CA) with a BigDye Cycle Sequencing kit (Applied Biosystems, Fostercity, CA). ITS sequences were generated in two independent sequencing reactions for each of the triplicates sampled for each species. No sequence divergence was observed within species, thus only one representative sequence of each species

was included in the phylogenetic analysis. The vouchers used for the genomic DNA extraction are as follows: *S. byzantina* (EA 4658, herb. Akçiçek), *S. thirkei* (EA 5209, herb. Akçiçek), *S. vuralii* (BY 16353, herb. Dirmenci).

Phylogenetic analysis

Alingment of the ITS sequences was generated using BioEdit (Hall 1999). ITS sequences of *S. byzantina* and *S. thirkei* were also searched with the BLAST program (Altschul *et al*. 1990) in the non-redundant nucleotide database of GenBank (NCBI), and the two most similar taxa to each were picked along with other (*Sideritis*) similar hits to construct a phylogenetic tree, which was inferred with the Neighbor-joining method (Saitou & Nei 1987) and constructed with MEGA4 software (Tamura *et al*. 2007). *Stachys germanica* subsp. *heldreichii* and *S. tmolea* both morphologically less similar to *S. vuralii* than *S. byzantina* and *S. thirkei* were included in the phylogenetic tree and sequence comparison.

Results

Stachys vuralii Yıldız, Dirmenci & Akçiçek, *sp. nova* (sect. *Eriostomum*) (Fig. 2)

Species Stachys byzantinae affinis, sed ab foliis oblongis versus ellipticis et discoloris (non oblonge-spathulatis versus lanceolatis et concoloris), rugosis, basi rotundatis ad subcordatis (non attenuatis vel raro rotundatis) calycis 5.5–8 mm longis (non 8–10 mm longis), calycis dentis valde recurvus et dense glandulosis (non erectis ad leviter recurvus et glandulosis vel raro sparsim glandulosis) differt.

HOLOTYPE: **Turkey**. A4 Bartın: Road from Bartin to Cide, 3 km W of Kurucasile, 41°50´12´´N, 32°42´10´´E, 100 m, *Pinus brutia* forest clearings, growing in calcareous gravel along roadside. 41°50´N, 32°42´E, 100 m, 4.VIII.2007 *Yıldız 16553*, *Dirmenci & Bräuchler* (holotype GAZI; isotypes C, EGE, G, HUB, ISTE, K, M, W, herb. Bräuchler).

PARATYPE: A4 Kastamonu: Road from Cide to Sinop, just W of Doganyol. 42°00´21´´N, 33°27´33´´E, 50 m. Cide, Doğanyol, *Pinus brutia* forest clearings, 50 m, 5.VIII.2007 *Yıldız 16556*, *Dirmenci & Bräuchler* (herb. Dirmenci, M and herb. Bräuchler keep isotypes and paratypes as well).

ETYMOLOGY: This species is named in honour of the eminent Turkish botanist Prof. Dr. Mecit Vural who is an expert of conservation biology of endemic plants in Turkey.

Perennial mesophytic herb, many-stemmed from base. Flowering stems 30–100 cm, usually branched above, rarely simple, densely adpressed tomentose to adpressed lanate-villous. Leaves 5–8 pairs per stem, 2–7 × 0.7–3 cm, oblong to broadly elliptic, diminishing from base to inflorescence, discolored, greenish and shortly sericeous-tomentose above; white floccose-tomentose beneath, crenulate, obtuse, rarely acute, rounded to subcordate at base, usually with 0.5–2.5 cm long petiole, except uppermost. Floral leaves sessile, lanceolate to linear, lower 1–3 times longer than verticillasters, upper shorter than verticillasters. Verticillasters (2–)3–9, lower ones distant to 2.5 cm, uppermost (2–3) usually congested, (8–)10–18 flowered. Bracteoles lanceolate to linear lanceolate, 2.5–5 mm, as long as or shorter than calyx tube, tip not spinescent, densely villose, sparsely glandular hairy. Pedicels 0.5–1.5 mm. Calyx 5.5–8 mm, sub-bilabiate, subcampanulate, densely villose and glandular papillate with sessile glands; mouth densely long villose; teeth 1.5–2.5 mm, triangular-lanceolate, 1/3–1/4 × tube, strongly recurved at and after anthesis, densely glandular papillate, tip spinescent. Corolla 10–12 mm, purplish-pink, tube slightly exserted from calyx, upper lip densely sericeous-tomentose outside, hairs longer than lip, lower lip 3-lobed, middle lobe much larger than lateral lobes; style not exceeding upper lip, glabrous, 2-lobed, lobes unequal; stamens 4, included in corolla; filaments villose towards thecae. Nutlets broadly obovate to ± rounded, faintly trigonous, 2–2.2 × 1.5–1.8 mm, slightly winged near base, glabrous, slightly tuberculate at apex, blackish-brown at maturity. Flowering and fruiting July–August.

DISTRIBUTION AND HABITAT ECOLOGY: *Stachys vuralii* is endemic to Bartın province (Fig. 1), north Anatolia, belonging in the Euro-Siberian element. It grows in *Pinus brutia* forest clearings at 100–230 m where a mixture of Euro-Siberian and Mediterranean elements is present. *Stachys vuralii* was growing with *Clinopodium nepeta* subsp. *glandulosum*, *Cistus creticus*, *Arbutus* sp., *Rubus* sp., *Sideritis* sp. and *Pteridium aquilinum*.

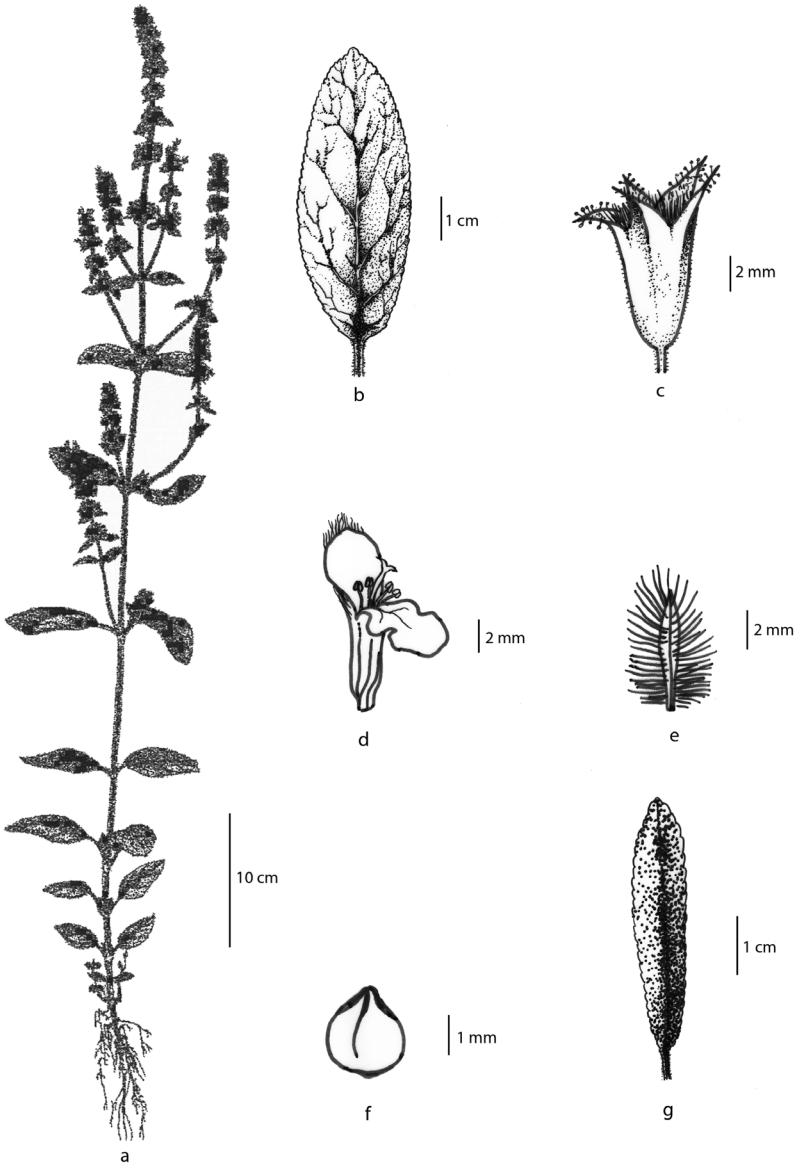

Fig. 2. *Stachys vuralii* (from the holotype). — **a**: Habit. — **b**: Cauline leaf. — **c**: Calyx. — **d**: Corolla. — **e**: Bracteole. — **f**: Nutlet. — **g**: *Stachys byzantina*, cauline leaf.

KARYOLOGY: *Stachys byzantina* (Fig. 3a), *S. vuralii* (Fig. 3b) and *S. thirkei* (Fig. 3c) have the somatic chromosome number (2n) of 30, suggesting the basic chromosome number (x) to be 15.

PHYLOGENETIC ANALYSIS AND DNA SEQUENCE COMPARISON: As can be seen from the DNA (ITS) sequence comparison (Fig. 4), *S. vuralii* differed from *S. byzantina* and *S. thirkei* in eight and nine nucleotides, respectively. BLAST search results revealed that the most similar two GenBank records for *S. byzantina* (this study) were *Sideritis glauca* (gi15429097) and *Sideritis algarviensis* (gi15429090), while *Sideritis tragoriganum* (gi15429106) and *Sideritis murgetana* (gi15429103) were the most similar to *Stachys vuralii*. Likewise, the most similar two GenBank records for *Stachys thirkei* (this study) were *Stachys hirta* (gi15429110) and *Sideritis discolor* (gi114796956). Hence the BLAST analysis revealed that *Stachys vuralii* was distinct. A phylogenetic analysis using all of the above-mentioned taxa along with *Phlomis lychnitis* (gi61098615) as an outgroup further confirmed the result (Fig. 4).

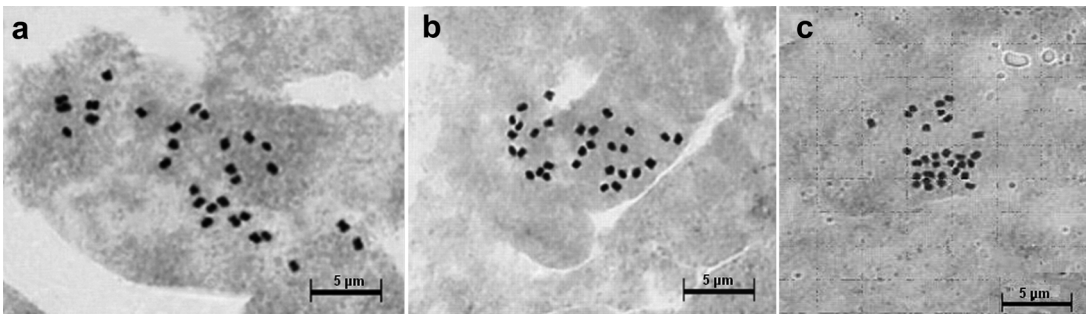

Fig. 3. Somatic metaphase chromosomes. — **a**: *Stachys byzantina* 2*n* = 2*x* = 30. — **b**: *Stachys vuralii* 2*n* = 2*x* = 30. — **c**: *Stachys thirkei* 2*n* = 2*x* = 30.

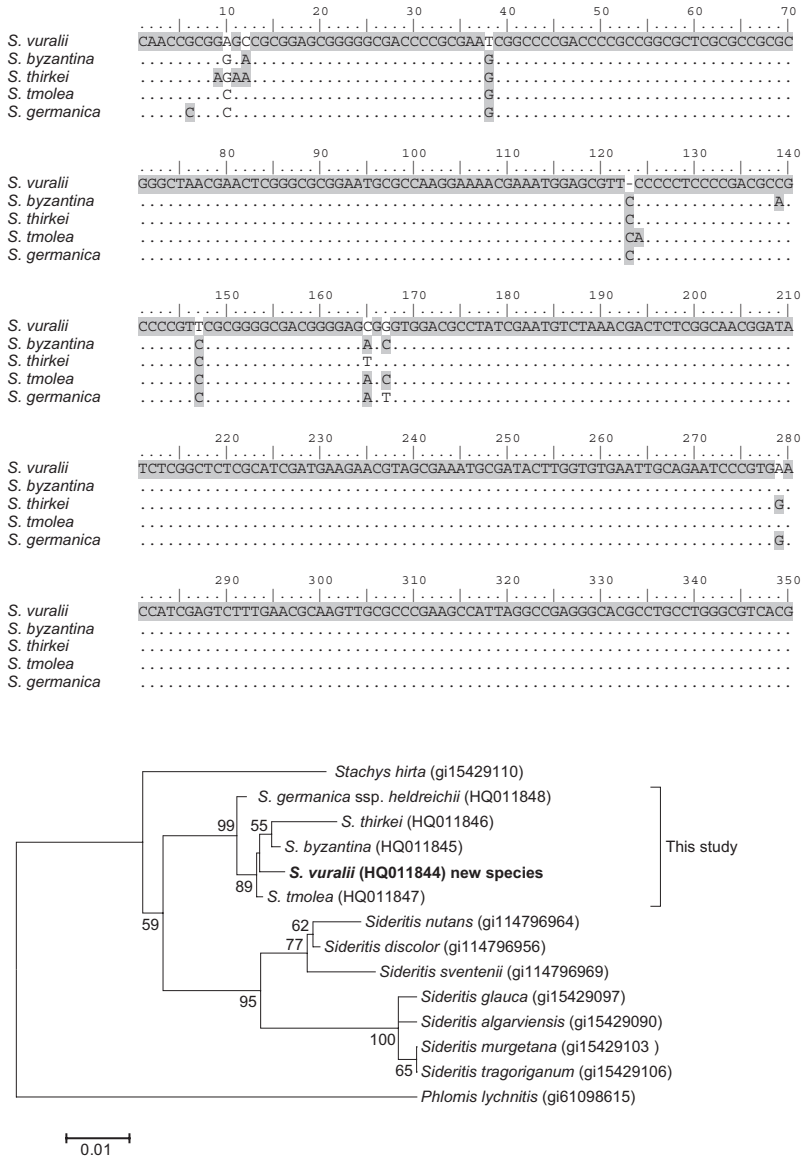

Fig. 4. Sequence alignment and phylogenetic analysis of *Stachys vuralii*. The upper panel shows the ITS sequence alignment of *S. vuralii* with *S. thirkei* and *S. byzantina* and with the less closely related *S. germanica* subsp. *heldreichii* and *S. tmolea*. Differing nucleotides are shown for each taxon. The alignment was constructed using BioEdit (Hall 1999). The lower panel shows the phylogenetic relationship of *S. vuralii*. The phylogenetic tree was inferred using the Neighbor-joining method (Saitou & Nei 1987). The bootstrap values (1000 replicates) are shown next to the branches (Felsenstein 1985). The phylogenetic distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004). There were a total of 346 nucleotides in the final dataset. *Phylomis lychnitis* was used as an outgroup. Accession numbers of the sequences obtained from GenBank are shown in parentheses and the sequences obtained in the present study are also shown in the upper panel.

Table 1. Morphological comparison of *Stachys vuralii, S. byzantina* and *S. thirkei*.

Characters	*S. vuralii*	*S. byzantina*	*S. thirkei*
Stem indumentum	densely adpressed tomentose to adpressed lanate-villous	densely lanate-villous to floccose-tomentose	densely grey tomentose to sparsely tomentose
Leaf shape	oblong to broadly elliptic	oblong-sphatulate to broadly lanceolate	oblong spathulate to broadly lanceolate
colour	discolourous	concolourous	concolourous
indumentum	shortly sericeous tomentose above, white floccose-tomentose below	densely sericeous tomentose to arachnoid on both surface	grey-tomentose to sparsely tomentose above, densely tomentose below
base	rounded to ± cordate	attenuate or rarely rounded at base	attenuate
Floral leaves	ovate to lanceolate, green	lanceolate, green	ovate, generally purplish at base
Calyx lenghth (mm)	5.5–8	8–10	8.5–12
indumentum	densely villous with glandular papillate	lanate-tomentose, eglandular or a few glandular	densely villose, glandular papillate
teeth	1/4–1/3 × tube, strongly recurved in flowering and fruiting time, densely glandular papillate	1/4–1/3 × tube, erect to slightly recurved, eglandular or a few glandular papillate	1/3–1/2 × tube, reflexed to clearly recurved in fruiting time, a few to many glandular papillate
Corolla length (mm)	10–12	12–14	12–15(–17.5)

Additional specimens examined: — *Stachys byzantina*: **Turkey**. Bilecik: *Akçiçek 4658 & Dirmenci* (herb. Akçiçek). Zonguldak: *Davis 37852*, *Coode & Yaltırık s.n.* (E, K). Kastamonu: *Davis 25057 & Polunin* (ANK, BM, K), *Davis 21781b* (K), *Jenkins 2201* (BM), *Huber-Morath 8392* (G), *B. Yıldız 16574 & Dirmenci* (herb. Dirmenci), *Davis 38651, Coode & Yaltırık* (E). Sinop: *B. Yıldız 16572 & Dirmenci* (herb. Dirmenci), *A. Dönmez 3687*, *H. Şağban & A. Kahraman* (W). Trabzon: *T. Baytop* (*ISTE-14264*). Konya: *Bornm. 5453* (G, K, W), *Huber-Morath 8392* (G). **Iran**. Mazanderan: *Rechinger f. 1993* (BM, K, W), *K.H. Rechinger 52400* (G), *Wendelbo 1108* (E, W), *N. Lindsay 1170* (BM, K). Gorgan: *Edmondson 702* (E, K, W), *Furse 7283* (K), *Furse 2848* (E). Gilan: *K. H. Rechinger 43363* (K, W). Otsan: *Schmid 6392* (W). **Azerbaijan**. Kaleybar: *Lamond 4807* (E, W). — *Stachys thirkei*: **Croatia**. *E. A. Mennega & W. G. Driehuis 35*, *42* (E). **Turkey**. İstanbul: *A. Baytop* (*ISTE-15557*) (W), *A. Berk & T. Baytop*, (*ISTE 3883*) (herb. Akçiçek), *Akçiçek 5075 & Dirmenci* (herb. Akçiçek), 7.VII.1902 *G. V. Aznavour s.n.* (G). Çanakkale: *Akçiçek 4541 & Dirmenci* (herb. Akçiçek). Bursa: *Bornm. 5451* (W), *Akçiçek 5214 & Dirmenci* (herb. Akçiçek). Bithynia: 26.V.1899 *Bornm. s.n.* (K), *Akçiçek 5209 & Dirmenci* (herb. Akçiçek). Kocaeli: *Huber-Morath 17245* (G). Bolu: *Davis 37129* & *Coode* (K), *Wagenitz & Beug 246* (W). Düzce: *Aslı Doğru Koca 1673* (G). Çankırı: *E. Erdoğan 1016 & S. Selvi* (herb. Akçiçek). Karabük: *M. Demirörs 1583* (ANK). Balıkesir: *A. Baytop & T. Avergil* (*ISTE 13712*) (ISTE). Kütahya: *Akçiçek 5210 & Dirmenci* (herb. Akçiçek). — *Stachys byzantina* × *S. vuralii* (putative hybrid): **Turkey**. Zonguldak: *Davis 38839* & *Coode* (E, K).

Discussion

Stachys vuralii resembles *S. byzantina* and *S. thirkei*, but differs from both in several characters (Table 1). Bhattacharjee (1982) indicated a specimen (*Davis 38839*) of *Stachys* collected from the Zonguldak province as a potential hybrid involving *S. byzantina*. Investigation of the specimen revealed that it is similar to *S. vuralii* by its ±cordate leaf base, rugose leaf surface and discoloration, while it resembles *S. byzantina* in having a densely lanate-villose to floccose tomentum on the stem, leaves and inflorescence, and ±congested verticillasters. Also, no pollen or seeds in the Zonguldak specimen from the Davis collection were observed. The collection might represent a hybrid or intermediate of *S. vuralii* and *S. byzantina*.

A karyological analysis (revealing a basic chromosome number x = 15 for *S. thirkei*, *S. byzantina* and *S. vuralii*) did not differentiate these taxa. A phylogenetic analysis and DNA (ITS) sequence comparison of *S. vuralii* with *S. byzantina* and *S. thirkei* along with other species (including *S. hirta*, *S. germanica* and *S. tmolea*), however, clearly supported *S. vuralii* as a distinct species (Fig. 4).

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