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Molecular phylogeny of *Scutellaria* (Lamiaceae; Scutellarioideae) in Iranian highlands inferred from nrITS and *trnL-F* sequences

Sareh Seyedipour[1], Yasaman Salmaki[1]*, Chun-Lei Xiang[2]

[1] *Center of Excellence in Phylogeny of Living Organisms and Department of Plant Science, School of Biology, College of Science, University of Tehran, P.O. Box 14155-6455, Tehran, Iran*

[2] *CAS Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China*

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ABSTRACT

Scutellaria with about 360 species is one of the largest genera of Lamiaceae. The Iranian highlands accommodate about 40 *Scutellaria* spp., and is considered as one of the main centers of diversity of the genus. Here, we present a phylogenetic study for 44 species of *Scutellaria* especially from Iranian highlands, representing major subgeneric taxa, based on nuclear ribosomal ITS and *trnL* intron and *trnL-F* intergenic spacer using Maximum Parsimony (MP) and Bayesian Inference (BI) analyses. The monophyly of *Scutellaria* is confirmed in our study, but *Scutellaria* subg. *Scutellaria* is shown to be paraphyletic with *S.* subg. *Apeltanthus* embedded within it. Moreover, our results reveal that *S.* subg. *Apeltanthus* is paraphyletic including one accession of *S. repens* of *S.* subg. *Scutellaria* nested within. In addition, the two sections of *S.* subg. *Apeltanthus*, i.e. sect. *Apeltanthus* and sect. *Lupulinaria*, are not supported as monophyletic by our plastid and nuclear topologies. Thus, the subgeneric classification of *Scutellaria* which is mainly based on morphological characters such as the type of inflorescence, shape of calyces, presence of a scutellum and a bladder-like appendage on the upper calyx lip is not supported by our molecular data. Additionally, our phylogenetic study corroborates Paton's finding on primitive position of *S.* sect. *Scutellaria*, but disagrees with the intermediate position of *S.* sect. *Salviifolia* between *S.* subg. *Scutellaria* and *S.* subg. *Apeltanthus*.

Keywords: Labiatae; Nuclear marker; Plastid marker; Subgeneric classification; Systematics

Introduction

With five genera (*Holmskioldia* Retz., *Renschia* Vatke, *Scutellaria* L., *Tinnea* Kotschy ex Hook.f., and *Wenchengia* C.Y.Wu & S.Chow) and about 380 species, Scutellarioideae is one of the 12 subfamilies currently recognized in Lamiaceae (1-2). *Scutellaria* alone comprises 360 species and is the largest genus of

* **Corresponding author:** ysalmaki@ut.ac.ir

the subfamily Scutellarioideae (3). It is subcosmopolitan in distribution, can be found mainly in temperate regions, but poorly represented in moist tropical lowlands (4). *Scutellaria* species are mostly annual or perennial herbs or subshrubs, with cymes arranged in racemes or panicles and posterior lip of calyx usually folded to produce a scutellum (3, 5).

Several infrageneric classifications has been proposed in *Scutellaria* mainly using classical taxonomic approaches. Hamilton (1832) recognized three sections in the genus, viz. sect. *Lupulinaria* A.Hamilton, sect. *Stachymacris* A.Hamilton and sect. *Galericularia* A.Hamilton (6). The number of sections was increased later to five by adding sect. *Heteranthesia* Benth. and sect. *Maschalostachys* Benth. (7), but the same author (Bentham) reclassified the genus into three sections (8) including: sect. *Lupulinaria*, sect. *Heteranthesia* and sect. *Vulgares* Benth. Bentham's system has been largely followed by Briquet (9), but he recognized only two subgenera: *S.* subg. *Euscutellaria* Briq. (including the members of sect. *Lupulinaria*, sect. *Heteranthesia* and sect. *Vulgares*) and *S.* subg. *Scutellariopsis* Briq. without any sectional assignment. *Scutellaria* was divided into four subgenera: *S.* subg. *Euscutellaria* Briq. (including sect. *Lupulinaria*, sect. *Stachymacris* and sect. *Galericularia*), *S.* subg. *Anaspis* (Rech.f.) Juz., *S.* subg. *Apeltanthus* (Nevskyi ex Juz.) Juz., and *S.* subg. *Cystaspis* (Juz.) Juz. in 'Flora Iranica' (10). The most recent and widely used classification of *Scutellaria* and its allied genera classified the genus into two subgenera: *S.* subg. *Scutellaria* Briq. and *S.* subg. *Apeltanthus* (3, 4). The former is characterized by having one-sided inflorescence and flowers subtended by leaves or leaf-like bracts, while the latter has a four-sided inflorescence with decussate flowers subtended by cucullate bracts. *Scutellaria* subg. *Scutellaria* was further divided into five sections, viz. sect. *Scutellaria*, sect. *Anaspis* (Rech.f.) Paton, sect. *Perilomia* (Kunth) Epling, sect. *Salazaria* (Torrey) Paton and sect. *Salviifoliae* (Boiss.) J.R.Edm., while *S.* subg. *Apeltanthus* consists of two sections, viz. sect. *Apeltanthus* and sect. *Lupulinaria* (3-4, 11).

Recent phylogenetic studies support the monophyly of *Scutellaria* based on two nuclear ribosomal DNA regions (ITS and ETS) (12, 13). Furthermore, two major clades were identified: the first clade included three species of *S.* subg. *Scutellaria* (*S. galericulata* L., *S. diffusa* Benth., and *S. nuristanica* Rech.f.), which is sister to the remaining species of *Scutellaria*. The second clade consisted of two subclades. The first subclade contained *S. shweliensis* W.W.Sm., five species of *S.* subg. *Apeltanthus* (*S. stocksii* Boiss., *S. alpina* L., *S. supina* L., *S. nepetifolia* Benth. and *S. platystegia* Juz.), and five species of *S.* subg. *Scutellaria* (*S. likiangensis* Diels, *S. baicalensis* Georgi, *S. kingiana* Prain, *S. viscidula* Bunge, and *S. macrodonta* Hand.-Mazz. The second unresolved subclade included several species of *S.* subg. *Scutellaria* mainly distributed in China (12, 13). In a later work, based on nrDNA ITS and cpDNA *trnL-F* sequences, two main clades corresponding to the two subgenera *Scutellaria* and *Apeltanthus* were identified for the Iranian *Scutellaria* (14). In addition, the isolated position of *S. galericulata* (subg. *Scutellaria*; sect. *Scutellaria*) from other species of sect. *Scutellaria* in both trees confirmed the placement of this species in the sect. *Galericularia* as previously considered by several authors (5, 6, 7, 10, 15).

Iranian highlands, an area including Iran, Afghanistan, W Pakistan, N Iraq, Azerbaijan, and Turkmenistan, is home to about 40 *Scutellaria* spp. and is one of the main centers of diversity of the genus (10). As a main part of the Irano-Turanian floristic region with a complex biogeographic history, the flora of Iranian highlands is rich in species, especially the endemic ones (16). Most species of *Scutellaria* in this area belong to *S.* subg. *Apeltanthus* sect. *Lupulinaria*, which is a taxonomically complicated group (17) but not well-represented in former molecular phylogenetic studies (12, 13).

The aim of the present study is to provide a phylogenetic backbone for the assessment of phylogenetic relationships among species of *Scutellaria* in Iranian highlands. Here, we also present a phylogenetic analysis based on nuclear (nrITS) and plastid (*trnL* intron and *trnL-F* intergenic spacer) DNA sequences to evaluate the current subgeneric classification of *Scutellaria*.

Materials and methods

Taxon sampling

All taxon names in the present study follow the *World*

Checklist of Lamiaceae & Verbenaceae (18). A total of 113 DNA sequences representing 44 species were generated from specimens held at the following herbaria: B, KUN, M and TUH. We present a phylogenetic study based on sequence data of two plastid regions (*trnL* intron and *trnL-trnF* intergenic spacer) as well as one nuclear ribosomal DNA region (ITS: ITS1, 5.8S rDNA, ITS2). The sampling strategy was to include as many *Scutellaria* species from the Iranian highlands as possible representing the four subgenera mentioned in 'Flora Iranica' (10) as well as the two subgenera recognized later (3). Only one species representing *S.* subg. *Cystapis* (10) is missing in our study, because no material was available. The sampled taxa represent almost all morphological lineages recognized already in *Scutellaria* in the Iranian highlands. *Rubiteucris palmata* (Benth. ex Hook.f.) Kudô, *Ajuga reptans* L., *Clerodendrum thomsoniae* Balf.f, *Teucrium orientale* L. (subfamily Ajugoideae, 4 spp.), *Holmskioldia sanguinea* Retz., *Tinnea rhodesiana* S.Moore (subfamily Scutellariodeae, 2 spp.), were selected as outgroups according to previous studies (12, 19). A list of all taxa included in this study and their summarized sources, voucher specimen data, and GenBank accession numbers of the sequences generated is given in Table 1.

DNA extraction, amplification and sequencing

The ITS region of nuclear DNA and partial *trnL* intron and *trnL-F* intergenic spacer from plastid DNA were selected as appropriate markers. Total genomic DNA was extracted from dried leaf material using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany). Protocols followed those provided by the manufacturer, except for an additional extraction step with phenol/chloroform to remove potentially interfering secondary compounds as explained earlier (20). The extracted DNA was resuspended in 50 µl elution buffer (10 mM Tris-HCl), and a standard amount of 1 µl of the solution was used for amplification (higher amounts up to 3 µl in cases where PCR yielded insufficient amounts of product). The markers were amplified from total DNA using *Taq*-polymerase (AGS, Heidelberg, Germany).

Amplification of the ITS region was conducted using the primers Leu1 (21), ITS4 (22), as well as ITS2 and ITS3 (22; see Table 2) in some difficult cases as described previously (23). The *trnL-F* region was amplified according to a study on *Stachys* (24), using the universal primers of Taberlet *et al.* (25; see Table 2). For amplifying the ITS and *trnL-F* regions from very old herbarium specimens, Phusion polymerase (New England Biolabs, Ipswich, Massachusetts, U.S.A.) was used as explained earlier (23). All PCR amplifications were carried out in a thermocycler type Primus 96 plus (MWG-Biotech, Ebersberg, Germany). Successful PCR reactions were purified with the NucleoSpin Extract II-Kit (Macherey-Nagel) following the manufacturer's instructions. Cycle Sequencing was carried out using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) in a final volume of 20 µl. Runs were performed on an ABI 3730 48 capillary sequencer (Applied Biosystems). In all cases, the markers were sequenced bi-directionally using the same primers as in PCR reactions.

Data matrix, alignment and phylogenetic reconstruction

All sequences generated in this study were assembled, edited, and aligned manually using Mesquite v.1.12 (26). The combined plastid markers and the nrITS dataset were analyzed separately. Phylogenetic reconstructions were performed with Bayesian Inference (BI), and Maximum Parsimony (MP) approaches. An alignment of nrITS with 58 accessions and a combined plastid alignment with 57 accessions were analyzed. Bayesian analyses were conducted using the Markov-Chain-Monte-Carlo algorithm of MrBayes v.3.1.4 (27) for 10,000,000 million generations. The used substitution models were those estimated as optimal using the Akaike information criterion (AIC) in jModelTest v.0.1.1 (28). The GTR+G was the estimated best-fit model for both combined plastid markers and nrITS. Trees were sampled every 1000th generation with the default of three "heated" and one "cold" chain. Burnin was set to 2500 in both analyses. The remaining trees were summarized in a 50% majority-rule consensus tree. Maximum parsimony analyses were performed on both datasets (ITS and combined plastid DNA) using PAUP* v.4.0b10 (29) with the following parameters: all characters unordered and equally weighted;

heuristic search with random sequence addition, tree-bisection-reconnection branch-swapping, 50 random-addition-sequence replicates, and MAXTREES option set to 10,000. Bootstrapping was done using the following settings: hsearch addseq=random, nchuck= 10, chuckscore=1, nreps=50, bootstrap nreps= 1000 (summarized in a 50% majority-rule consensus tree as a cladogram).

Table 1. Voucher information (only for species with new sequences) and GenBank accession numbers of studied taxa are given for ITS and *trn L* intron/*trn L-F* intergenic spacer. GenBank accession numbers for sequences from previous studies are provided with reference to place of original publication. References are indicated by running numbers following the corresponding accession number and are as follows: (1) Scheen et al., 2007; (2) Yuan et al., 2009; (3) Bendiksby et al., 2011; (4) Wang et al., 2011; (5) Xiang et al., 2012; (6) Chen and Chen, 2012; (7) Xia, 2013; (8) Xu, 2015; (9) Son and Park., 2015; (10) Salmaki et al., 2016; (11) Zhao et al., 2017; (12) Xiang et al., 2018; (13) Salmaki and Müller. An asterisk denotes a new sequence; and Missing data are indicated as N/A.

Subgenus	Section	Species	Collection data	ITS	*trn*L intron/ *trn*L-F spacer
OUTGROUPS					
		Ajuga reptans L.		JN575347 (10)	JN408587 (10)
		Clerodendrum thomsoniae Balf.f.		JN575348 (10)	JN408588 (10)
		Holmskioldia sanguinea Retz.		JQ618372 (7)	JX893333 (5)
		Holmskioldia sanguinea Retz.		MF193548 (11)	N/A
		Rubiteucris palmata (Benth. ex Hook.f.) Kudô (1)		JN575349 (10)	JN575438 (10)
		Rubiteucris palmata (Benth. ex Hook.f.) Kudô (2)		MF801679 (12)	
		Teucrium orientale L.		JN575413 (10)	JN408651 (10)
		Tinnea barbata Vollesen	South Africa: Pietermaritz-burg, G. Stafford, GIS-357 (KUN)	N/A	**MT265260**
		Tinnea galpinii Briq.	South Africa: Pietermaritz-burg, G. Stafford, GIS-358 (KUN)	N/A	**MT265261**
		Tinnea rhodesiana S. Moore	South Africa: Pietermaritz-burg, G. Stafford, GIS-359 (KUN)	MF193549 (11)	**MT265262**
INGROUPS					
Scutellaria subg. *Apeltanthus*	*Apeltanthus*				
		Scutellaria immaculata Nevski ex Juz.	Russia: Uzbekistan, Tianschan, M. Vatolkina, 6757 (B)	**MT249824**	**MT265263**
		Scutellaria leptosiphon Nevski	Afghanistan: Mazar-e Sharif, K.H. Rechinger, 16220 (B)	**MT249825**	**MT265264**
		Scutellaria stocksii Boiss.	Afghanistan: Gardes, montes Safed kuh, K.H. Rechinger, 31949 (B)	**MT249826**	N/A
		Scutellaria stocksii Boiss.	Iran: Anonymous, 30348 (TUH)	MF193543 (12)	**MT265265**
	Lupulinaria				
		Scutellaria alpina L.	Europe alpine region: P.C. Liao, s.n. (KUN)	MF193544 (11)	**MT265266**
		Scutellaria araxensis Grossh.	Iran: W. Azarbaijan, S. Khoy, K.H. Rechinger, 41797 (B)	**MT249827**	N/A
		Scutellaria farsistanica Rech.f.	Iran: W-Qashqai, Kuhruyeh, K.H. Rechinger, 47342 (B)	**MT249828**	**MT265267**
		Scutellaria glechomoides Boiss. ex Benth.	Iran: N. Elburz, A. Bornmüller, 8048 (B)	**MT249829**	**MT265268**

Table 1. Continued.

Subgenus	Section	Species	Collection data	ITS	*trn*L intron/ *trn*L-F spacer
		Scutellaria karjaginii Grossh.	Caucasus: Ararat, montes Gegamski Khrebert, V. Vašák, s.n. (B)	**MT249830**	**MT265269**
		Scutellaria linearis Benth.	Afghanistan: Khost, K.H. Rechinger, 35513 (B)	**MT249831**	**MT265270**
		Scutellaria litwinowii Bornm.	Iran: Khorasan, K.H. Rechinger, 51098 (B)	**MT249832**	**MT265271**
		Scutellaria luteocaerulea Bornm. & Sint.	Iran: Khorasan, Shah Abad to Bojnurd, K.H. Rechinger, 55499 (B)	**MT249833**	**MT265272**
		Scutellaria multicaulis Boiss.	Afghanistan: Kabul, I. Hedge, 17116 (B)	**MT249834**	**MT265273**
		Scutellaria nepetifolia Benth. (1)	Iran: Lorestan, Khoramabad, Sefid Kouh, G. Veisekarami, 23940 (TUH)	MF193545 (11)	**MT265274**
		Scutellaria nepetifolia Benth. (2)	Iran: W. Azarbaijan, K.H. Rechinger, 48882 (B)	**MT249835**	**MT265275**
		Scutellaria persica Bornm.	Iran: SW. Zanjan (Khamseh) to Bijar, K.H. Rechinger, 42405 (B)	**MT249836**	**MT265276**
		Scutellaria pinnatifida A.Ham.	Iran: E. Azarbaijan, Y. Salmaki & S. Siadati, 33365 (TUH)	**MT249837**	**MT265277**
		Scutellaria platystegia Juz.	Iran: Azarbaijan, A. Ghahraman, 7697 (TUH)	MF193546 (11)	**MT265278**
		Scutellaria przewalskii Juz.	Kyrgyzstan: Tianschan, A. Dürbay, 1755 (B)	**MT249838**	**MT265279**
		Scutellaria supina L.		JX893233 (5)	JX893337 (5)
		Scutellaria theobromina Rech.f.	Iran: W. Azarbaijan, SW. Rezaieh, K.H. Rechinger, 49306 (B)	**MT249839**	N/A
		Scutellaria tomentosa Bertol.	Iran: Khashan, K.H. Rechinger, 46863 (B)	**MT249840**	**MT265280**
Scutellaria subg. *Scutellaria*	*Anaspis*				
		Scutellaria ariana Hedge	Afghanistan: D. Podlech, s.n. (B)	MK561740 (13)	**MT265281**
	Salvifolia				
		Scutellaria diffusa Benth.	Turkey: H. Ern, 6923 (B)	MK561741 (13)	**MT265282**
	Scutellaria				
		Scutellaria albida L.	Turkey: D. Tolimir, 1644 (B)	MK561742 (13)	N/A
		Scutellaria baicalensis Georgi		MF193525 (11)	GQ374139 (5)
		Scutellaria barbata D.Don.	China: Beijing, C.L. Xiang, 282 (KUN)	MF193539 (11)	**MT265283**
		Scutellaria discolor Wall. ex Benth.	China: Yunnan, C.L. Xiang, 438 (KUN)	MF193504 (11)	**MT265284**
		Scutellaria franchetiana H.Lev.	China: Yunnan, C.L. Xiang, 287 (KUN)	MF193532 (11)	**MT265285**
		Scutellaria indica Roxb.	China: Hongkong, H. Peng, s.n (KUN)	MF193513 (11)	**MT265286**
		Scutellaria megalaspis Rech.f.	Iraq: K.H. Rechinger, 10866 (B)	MK561743 (13)	**MT265287**
		Scutellaria regeliana Nakai	China: Neimenggu, L. Jiang, 149 (KUN)	MF193536 (11)	**MT265288**
		Scutellaria repens Buch.-Ham. ex D.Don	Pakistan: NW. Frontier, H. Ern, 7539 (B)	**MT249841**	**MT265289**
		Scutellaria scordifolia Fischer ex Schrank.	China: W.T. Yu et al., 2822 (KUN)	MF193540 (11)	**MT265290**
		Scutellaria shweliensis W.W.Sm.	China: F. Zhao et al., ZF0068 (KUN)	MF193530 (11)	**MT265291**
		Scutellaria sieberi Benth. (1)		N/A	EF546928 (7)
		Scutellaria sieberi Benth. (2)		N/A	EF546848 (7)

Table 1. Continued.

Subgenus	Section	Species	Collection data	ITS	*trn*L intron/ *trn*L-F spacer
		Scutellaria tournefortii Benth.	Iran: Mazandaran, Y. Salmaki, H. Moazzeni & A. Pirani, s.n. (TUH)	MK561745 (13)	**MT265292**
		Scutellaria xylorrhiza Bornm.	Iran: Isfahan, Sofeh mountain, Y. Salmaki & S. Zarre, 45417 (TUH, M)	MK561744 (13)	**MT265293**
		Scutellaria yunnanensis H.Lev.	China: Yunnan, C.L. Xiang, 547 (KUN)	MF193507 (11)	**MT265294**
Unplaced		*Scutellaria caryopteroides* Hand.-Mazz.	China: C.L. Xiang, 321 (KUN)	KC535536	**MT265295**
		Scutellaria chorassanica Bunge	Iran: Semnan, Turan protected area, SE. Shahrud, H. Freitag, 14881 (B)	**MT249842**	**MT265296**
		Scutellaria hirta Sm. (1)		N/A	EF546847 (7)
		Scutellaria hirta Sm. (2)		N/A	EF546927 (7)
		Scutellaria kingiana prain	China: Xizang, J.W. Zhang et al., ZJW-3890 (KUN)	MF193542 (11)	**MT265297**
		Scutellaria likiangensis Diels.	China: Yunnan, C.L. Xiang et al., 373 (KUN)	MF193524 (11)	**MT265298**
		Scutellaria macrodonta Hand.-Mazz.	China: Beijing, F. Zhao et al., 2015-006 (KUN)	MF193523 (11)	**MT265299**
		Scutellaria pekinensis Maxim.		KC535534 (7)	N/A
		Scutellaria rehderiana Diels (1)		JX893232 (5)	JX893338 (5)
		Scutellaria rehderiana Diels (2)		N/A	JN675928 (4)
		Scutellaria tenera C.Y.Wu & H.W.Li	China: Jiangxi, Y.P. Chen et al., EM187 (KUN)	MF193522 (11)	**MT265300**
		Scutellaria tsinyunensis C.Y.Wu & S.Chow	China: C.L. Xiang, 519 (KUN)	KU365157 (8)	**MT265301**
		Scutellaria viscidula Bunge		MF193526 (11)	JN675929 (4)

Results

Detailed information about alignment characteristics and MP statistics are summarized in Table 3. Results from the BI were largely congruent with those from the MP analysis. Since the results from the BI receive better topological resolution and overall higher branch support, only the Bayesian 50% majority-rule consensus trees of the ITS dataset (Figure 1) and the combined *trnL* intron and *trnL-F* intergenic spacer dataset (Figure 2) are presented.

The length of ITS sequences ranged from 683 bp (in *Ajuga reptans* and *Clerodendrum thomsoniae*) to 796 bp (in *S. megalaspis* and *S. albida*), while the length of *trnL* intron + *trnL-F* intergenic spacer ranged from 485 bp (in *S. tomentosa*) to 892 bp (in *S. hirta* and *S. sieberi*). Corresponding to the larger number of informative characters, resolution of branches in the nrITS phylogeny was slightly higher than in the plastid phylogeny. Therefore, we perform our discussion mainly based on the nuclear tree, while relationship inferred from the plastid data will only be discussed where relevant. Almost the same monophyletic groups were recovered in the ITS tree (Figure 1) and plastid tree (Figure 2). All trees obtained from nrITS and plastid markers were congruent in showing *Scutellaria* as monophyletic with high support (PP=1.00, BS=94% in Figure 1; PP=1.00, BS=95% in Figure 2).

In the ITS phylogeny (Figure 1), two major lineages were recovered in *Scutellaria*. The first lineage (Clade A; PP=1.00, BS=100%) is a polytomic assemblage of *S. albida*, *S. megalaspis*, *S. diffusa* and members of *Galericulata* clade (including *S. ariana*, *S. xylorrhiza*, *S. tournefortii* and *S. galericulata*). The second lineage (Clade B–E; PP=0.92, BS=76%), which is the most diverse group, included representatives of both subgenera of *Scutellaria*, and consists of four main clades: (1) Clade B (PP=0.99, BS=94%), including several species of *S. scordifolia* alliance was sister to the remaining species of *Scutellaria*; (2) Clade C (PP=1.00, BS=93%)

Table 2. Primers used to amplify and sequence rDNA and cpDNA.

Marker	Primer name	Sequences	References
ITS	Leu1	5'-GTCCACTGAACCTTATCATTTAG-3'	Vargas & al. (1998)
	ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	White & al. (1990)
	ITS3	5'-GCATCGATGAAGAACGCAGC-3'	White & al. (1990)
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White & al. (1990)
***trnL* intron + *trnL-F* intergenic spacer**	trnL (UAA) F (TabC)	5'-CGAAATCGGTAGACGCTACG-3'	Taberlet & al. (1991)
	trnF (GAA) F (TabF)	5'-ATTTGAACTGGTGACACGAG-3'	Taberlet & al. (1991)
	trnL (UAA) R (TabD)	5'-GGTTCAAGTCCCTGATCCC-3'	Taberlet & al. (1991)
	trnL (UAA) R (TabE)	5'-GGTTCAAGTCCCTCTATCCC-3'	Taberlet & al. (1991)

Table 3. Various sequence alignment information and tree statistics. Abbreviations: ASDSF = average, standard deviation of split frequencies, bp=base pairs, CI=Consistency Index (Kluge and Farris, 1969), RI = Retention Index (Farris, 1989), MPT=most parsimonious tree, GTR=general time reversible, G=gamma distribution, TVM=transversion model.

	***trnL* intron + *trnL-F* intergenic spacer**	**ITS1 + 5.8s + ITS2**
Number of taxa	57	58
Sequence length [bp]	485–892	683–764
Aligned length [bp]	893	778
Constant characters [bp]	591	460
Variable but parsimony-uninformative characters [bp]	189	107
Parsimony-informative characters [bp]	113	211
CI of MPTs	0.890	0.629
CI of MPTs (excluding uninformative characters)	0.758	0.559
RI of MPTs	0.889	0.809
Length of MPTs	411	818
Selected substitution model	GTR+G[a]	GTR+G
ASDSF at termination	0.0071	0.0049

A: jModelTest selected TVM+G as the best-fit model for the cpDNA dataset. This model of evolution, characterized by a five-parameter nucleotide substitution rate matrix, is not currently available in MrBayes. Instead, we used the model that was selected as the second best: the parameter rich GTR + G.

contained eight species of *S.* subg. *Scutellaria* as well as some taxonomically unassigned species mostly from China; (3) Clade D (PP=0.99, BS=63%) embraced the members of *S. baicalensis* alliance, and is sister to Clade E; (4) Clade E (PP=0.98, BS=53%) comprised all species of *S.* subg. *Apeltanthus* along with *S. repens* Buch.-Ham. ex D.Don of *S.* subg. *Scutellaria*. Clade E, which is mostly confined to the Iranian highlands, is divided into two species groups: (1) *S. multicaulis* Boiss. alliance which is sister to remaining members of this clade, and (2) *Scutellaria* core group, which splits into two subclades: *S. stocksii* Boiss. and *S. persica* Bornm. alliances. Although the monophyly of clade E is highly supported in the ITS tree (Figure 1), in the plastid trees (Figure 2) the species of this unresolved clade form a polytomy with members of clade D in the plastid tree (Figure 2). In addition, *S. shweliensis* did not place in any further subclade within Clade E (Figure 1).

In the combined plastid trees almost the same groups as in nrITS tree were recovered, but some differences were observed in species placements and support values. The plastid phylogeny consisted of two major lineages: the first lineage contained Clades B and C, the latter being strongly supported (PP=1.00, BS=100%), and the second lineage was comprised of Clades A, D and E (PP=0.91, BS=65%).

Discussion

Our molecular phylogenetic analyses based on both nuclear and plastid DNA sequences, show that *Scutellaria* is monophyletic, which corroborates previous investigations (12, 13) that provided a limited taxonomic coverage. The inclusion of 44 species of *Scutellaria* in the present study reveals several clades not previously evident. Moreover, the use of both nuclear and plastid data enabled us to identify several instances of nuclear-plastid incongruence. Almost all phylogenetic analyses indicate similar groups of species in each clade (Figures 1-2, Clades A-E), but relationships among these groups differ in the plastid and the nuclear trees. This difference in topology may be caused either by past hybridization events or lack of

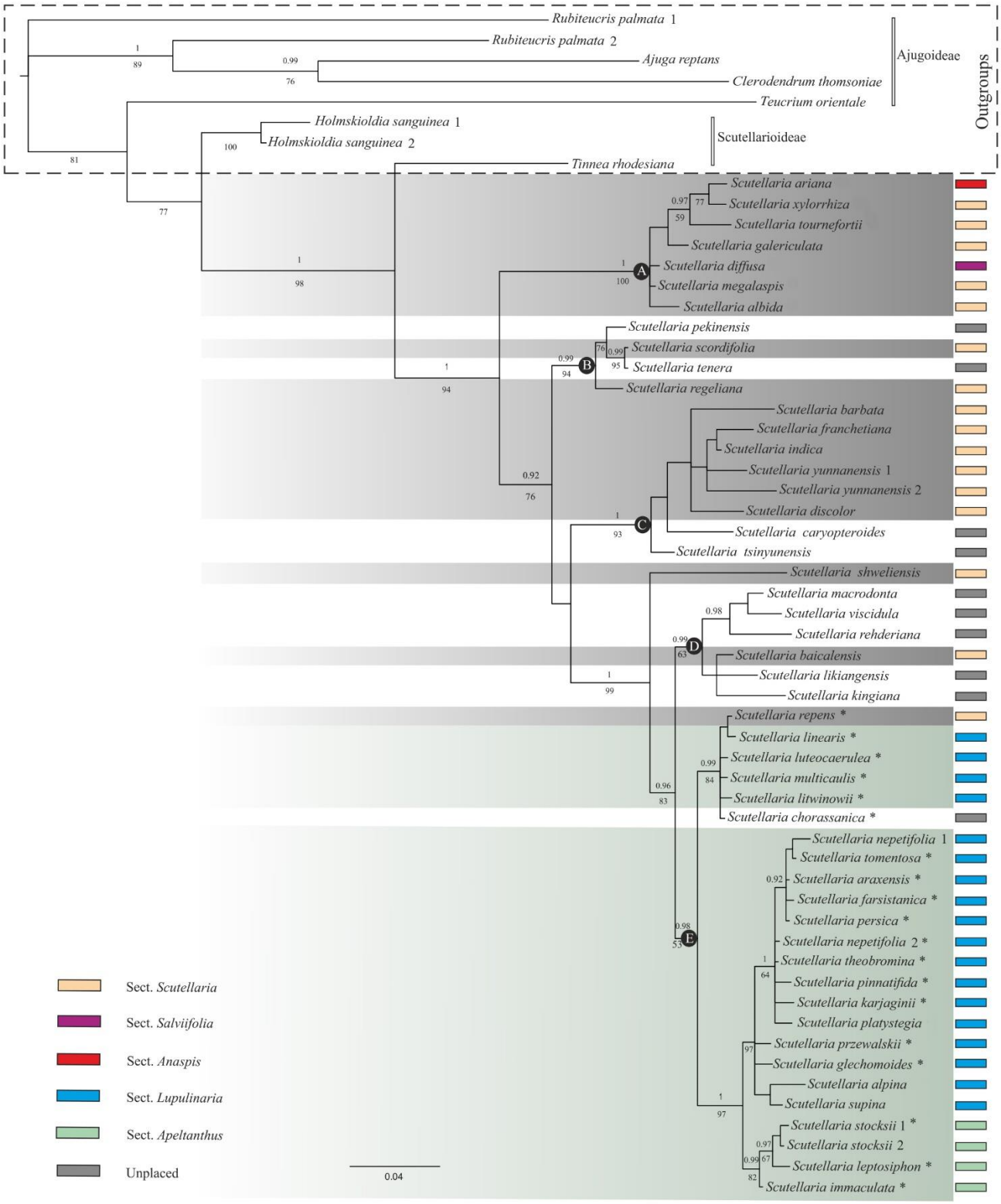

Figure 1. Strict consensus tree inferred from Bayesian analysis of the ITS dataset. Posterior probabilities (PP) equal to or greater than 0.90 are given above each node, corresponding bootstrap support (BS) values from a MP 50% majority-rule consensus tree (not shown) are indicated below each node. The newly generated sequences for the present study are indicated with an asterisk. *Scutellaria* subg. *Scutellaria* and *S.* subg. *Apeltanthus* are indicated by gray and green background colors on tree, respectively.

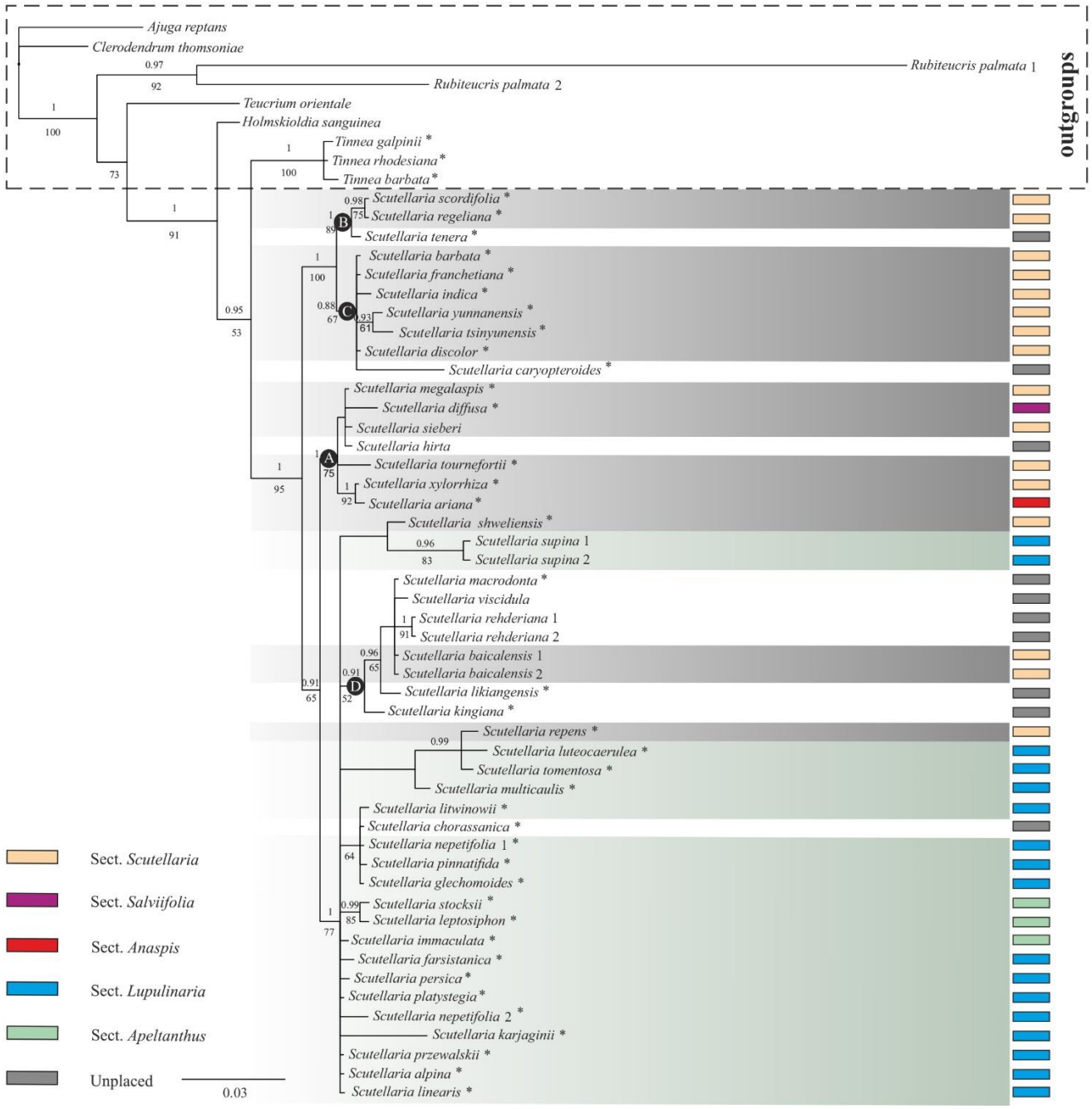

Figure 2. Strict consensus tree inferred from Bayesian analysis of the combined plastid dataset. Posterior probabilities (PP) equal to or greater than 0.90 are given above each node, corresponding bootstrap support (BS) values from a MP 50% majority-rule consensus tree (not shown) are indicated below each node. The newly generated sequences for the present study are indicated with an asterisk. Subgenus Scutellaria and subgenus Apeltanthus are indicated by gray and green background colors on tree, respectively.

sufficient informative signal in the *trnL-F* region. However, most of the topological differences between nuclear and plastid trees, can be considered as "soft incongruence" and will likely be resolved by the use of additional markers (30). In general, our results do not support the current infrageneric classification proposed by Paton (3, 11) which divided *Scutellaria* into two subgenera using inflorescence morphology, as well as characteristics of corolla, calyx, bracts, and nutlets.

Scutellaria *subg.* Apeltanthus

Following morphological characters support *S.* subg.

Apeltanthus as a reliable taxonomic group: 4-sided inflorescence as well as flowers opposite and decussate subtended by cucullate bracts. Most species of *S.* subg. *Apeltanthus* are distributed in Iranian highlands and occupy dry mountainous habitats, rock crevices, screes, and steppes (3). Contrary to Safikhani et al. (13) finding *S.* subg. *Apeltanthus* monophyletic (Clade II; 13), our ITS phylogeny reveals that *S.* subg. *Apeltanthus* is paraphyletic including a clade with one accession of *S. repens* of *S.* subg. *Scutellaria* nested within (Figure 1, Clade E). *Scutellaria repens* and three other species (i.e. *S. sessilifolia* Hemsl., *S. scandens* D.Don, and *S. franchetiana* H.Lév.) have been assigned to the "*S. repens* species group" in *S.* subg. *Scutellaria* (3). Although *S. sessilofolia* and *S. franchetiana* are placed in different clades together with other representatives of *S.* subg. *Scutellaria* (Figure 1, Clade C), *S. repens* shows a close relationship with members of *S.* subg. *Apeltanthus*. However, Paton (3) mentioned that the nutlets of *S. repens* which are completely covered with hairs on surface, are similar to *S.* subg. *Apeltanthus* sect. *Lupulinaria*. Our molecular phylogenetic results show that *S. repens* should not be treated as a member of *S.* subg. *Scutellaria*, but a close relationship with *S.* sect. *Lupulinaria* could not be evaluated here. The phylogenetic placement of this species awaits further analyses with inclusion of more taxa of this group.

Clade E contains two well-supported subclades including: 1- several members of *S.* sect. *Lupulinaria* as well as *S. repens*, and 2- the remaining species of *S.* sect. *Lupulinaria* as well as the majority of *S.* sect. *Apeltanthus*. Thus, corroborating previous phylogenetic studies (13) non-monophyly of sect. *Apeltanthus* is confirmed here and the division of *S.* subg. *Apeltanthus* into two sections, i.e. *S.* sect. *Apeltanthus* and *S.* sect. *Lupulinaria*, as suggested by Paton (3) is not supported by our plastid and nuclear topologies. In addition, several cases of incongruence were observed among components of Clade E in nuclear and plastid phylogenies (Figures 1-2). Since only nrITS and *trnL-F* sequences were used here, probably the lack of sufficient informative signals is the main reason for this incongruence.

Recently, Safikhani et al. (17) have splitted Iranian *S. multicaulis* to several taxa, i.e. *S. patonii* Jamzad & Safikhani, *S. arakensis* Jamzad & Safikhani, and *S. multicaulis* subsp. *multicaulis* var. *gandomanensis* Jamzad & Safikhani. In addition, they recovered *S. patonii* in Clade IIA together with *S. linearis* and *S. litwinowii* (13). In the present study we examined one specimen of *S. multicaulis* from Afghanistan which is phylogenetically related to *S. luteocaerulea*, *S. linearis* and *S. litwinowii* (Clade E, Figure 1) representing an interesting case for arising the idea that *S. multicaulis* complex needs to be investigated more in depth and including more accessions from other countries.

Scutellaria *subg.* Scutellaria

This group of species is morphologically characterized by one-sided or rarely spiral inflorescences, flowers opposite or not, subtended by leaves or leaf-like bracts (3). Most species assigned to this subgenus are dispersed among several basal lineages (Figure 1, Clades A-D) geographically distributed in Central Europe to South West Asia (Syria and Turkey), Iranian highlands (Figure 1, Clade A) and East Asia with a diversity hotspot in China (Figure 1, Clades B-D), but it includes also a few species extending to the Mediterranean Europe. Our results show that *S.* subg. *Scutellaria* as defined previously (3) is paraphyletic (Figures 1-2, Clades A-D), however, *S.* subg. *Scutellaria* consists of a large number of Chinese taxa which are not included in Paton's classification (3).

The phylogenies presented here show that the well-supported Clade A comprises several species of *S.* subg. *Scutellaria* representing different sections, i.e. *S.* sect. *Scutellaria*, *S.* sect. *Anaspis*, and *S.* sect. *Salviifolia*. Although our phylogenetic study corroborates Paton's view (3) regarding the basal position of *S.* sect. *Scutellaria*, our results disagree with the intermediate position of *S.* sect. *Salviifolia* between the two mentioned subgenera.

Scutellaria diffusa and *S. salviifolia* were placed in *S.* sect. *Stachymacris* (31), but were later classified in *S.* sect. *Salviifoliae* by Edmondson (15) based on Boissier (32) who assigned them to *S.* subsect. *Salviifoliae*. *Scutellaria* sect. *Salviifoliae* is characterized by ovate and entire leaves which are well presented in *S. glechomoides*, an alpine Iranian endemic species of *S.* subg. *Apeltanthus* sect. *Lupulinaria*. Therefore, *S.* sect. *Salviifoliae* was

considered to provide a link between *S.* subg. *Apeltanthus* and *S.* subg. *Scutellaria* (3). The characteristic features of *S.* sect. *Salviifoliae* are prostrate and often mat-forming habit, small ovate entire leaves, and secund flowers which are arranged in a compact or elongated spike with small sessile bracts. However, *S. diffusa* which is supposed to be a close relative of *S. glechomoides* (3, 31), is recovered in a rather distant clade (Figures 1-2, *S. diffusa* in Clade A and *S. glechomoides* in Clade E). Thus, the morphological characters which correlate these species to each other seem to be plesiomorphic and cannot characterize monophyletic groups. In addition, the four-sided (vs. secund) inflorescence should be a synapomorphy for the majority of *S.* sect. *Lupulinaria*.

Based on our nuclear data, the only representative of *S.* sect. *Anaspis*, i.e. *S. ariana* Hedge, is sister to *S. xylorrhiza*, a very rare Iranian endemic (Figure 1; PP= 0.72, BS= 77%). Morphologically, *S. ariana* is similar to *S. xylorrhiza* in having suffruticose life form, living in rocky habitats, few or several slender stems arising from a thick woody rhizome, and blue corolla (33). However, in our ITS phylogeny, both of these chasmophytic species are placed close to *S. tournefortii*, a geophyte from Hyrcanian forests, with relatively high support (Figure 1, Clade A). The relationships among these species is unresolved in the plastid phylogeny (Figure 2).

Clade B is strongly supported in the ITS tree (Figure 1; PP= 0.99, BS= 94%), which was also recovered in a previous molecular phylogenetic study (13, 14). Most species of this clade (e.g. *S. scordifolia* and *S. regeliana*) were classified in *S.* sect. *Scutellaria* and have a distribution range extending from Russia to Mongolia, and East Asia.

Most species of the Clade C are widely represented in China (about 40 species), with a diversity hotspot in Yunnan and Sichuan. These species are typically recognized by one-sided inflorescence composed of secund flowers and leaf-like bracts. Consequently, they have been placed in *S.* sect. *Scutellaria* (3). However, *S.* sect. *Scutellaria* is not monophyletic based on our molecular phylogenetic results. Relationships among members of this clade are unresolved, since only nrITS (insufficiently informative) was used for analysis here. More nuclear and plastid DNA markers and a broader sampling will improve the resolution of the phylogeny on this group.

Although *S. baicalensis* belongs to the same species group as *S. regeliana* and *S. scordifolia* ("*S. strigillosa* species-group") (3), it is placed in a separate clade (Figure 1, Clade D) in our nrITS phylogeny. However, Paton (3) mentioned that *S. baicalensis* differs from other species of this group by having black nutlets.

Overall, the present study did not support Paton (3, 4) who proposed the most comprehensive infrageneric classification of the genus *Scutellaria* recognizing two major subgenera: *Apeltanthus* and *Scutellaria*. Clearly, more studies of various kinds (e.g., 34-36), are needed in order to reach the desired taxonomic update in to a more natural classification of *Scutellaria*.

Conclusion

The present study may be viewed as a preliminary but a sound starting point for future more in-depth studies that would shed light on the interpretation of relationships within *Scutellaria* that can be used for a systematic interpretation. However, a much larger sampling and additional DNA sequence data with high levels of variation would be required to ultimately address the infrageneric relationships of *Scutellaria*. The subgeneric division provided by Rechinger (10) and Paton (3, 4) which were based mainly on inflorescence type, calyx shape and presence or absence of a scutellum and a bladder-like appendage on the upper calyx lip, have not received adequate support from our molecular phylogenetic data. In addition, some of the unnamed clades within *Scutellaria* that are supported in the phylogeny could potentially represent taxonomically valid groups at sectional or subgeneric ranks. Thus, more studies are needed for a natural classification reflecting relationships among *Scutellaria* spp.

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Editorial Note

Volume 7, issue 2 of Progress in Biological Sciences was initialy scheduled to be published in December 31, 2017. However, some administrative changes leaded to a major delay in processing of the manuscripts. This issue is actually published in May 1, 2020. Editor-in-chief apologizes deeply for any inconvenience caused especially to the authors.