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Semiaquilegia danxiashanensis (Ranunculaceae), a new species from Danxia Shan in Guangdong, southern China

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Abstract

Based on morphological and molecular phylogenetic studies, *Semiaquilegia danxiashanensis*, a new species from Danxia Shan in northern Guangdong, southern China, is described and illustrated. This species is easily distinguishable from each of other three known species in the genus by characters of the flowers and fruits. In addition, molecular phylogenetic analyses of both the nuclear ITS and the plastid *trnL-F* region strongly supported *S. danxiashanensis* as a separate species from other species of *Semiaquilegia*. We provide a detailed morphological and habitat description, distribution, as well as colour photographs and illustrations of the new species.

Keywords: *Dichocarpum*, ITS, morphology, taxonomy, *trnL-F*

Introduction

The Danxia landform has been destination that attracts not only researchers (e.g. biologists) but also tourists due to its special natural and cultural scenery and resources (Huang 1999; Peng 2000; Zhou *et al.* 2016; Luo *et al.* 2017). This landform consists of red terrestrial clastic rock, which is characterized by its red cliffed scarp (Peng 2000; Lu *et al.* 2003). In China, the Danxia landform lies mainly in southern and southeastern regions and possesses high floristic endemism (Liu *et al*. 1999; Qi *et al*. 2005; Chen *et al.* 2008; Zhong *et al*. 2010; Yan *et al.* 2012; Huang *et al.* 2015). Numerous endemic new taxa, mainly in the Gesneriaceae (Shen *et al*. 2010; Guo *et al.* 2016; Zhou *et al.* 2016; Tian *et al.* 2018), Rubiaceae (Wang *et al.* 2015) and Orchidaceae (Zhai *et al.* 2013), have been recently discovered and reported from these areas.

During an expedition to Danxiashan National Nature Reserve in northern Guangdong, China, several peculiar populations of Ranunculaceae were discovered on the wet cliffs with dripping water. They appear to be similar to species of *Dichocarpum* Wang & Hsiao (1964: 323) in having clawed petals golden-yellow in the upper part and much smaller than the sepals. However, they are also morphologically similar to species of *Semiaquilegia* Makino (1902: 119) by having ca. five staminodes, usually four pistils and ellipsoid and densely rugose seeds. Based on survey of literature (Hsiao 1979; Fu & Orbélia 2001; Kadota 2006; Huang *et al.* 2017; Son *et al.* 2017), observations of herbarium specimens and living plants and particularly molecular phylogenetic analyses from both the plastid and nuclear DNA sequences, we determined that the populations in question represent a hitherto undescribed species of *Semiaquilegia*, which is described below as *S. danxiashanensis* (Figs. 1–3).

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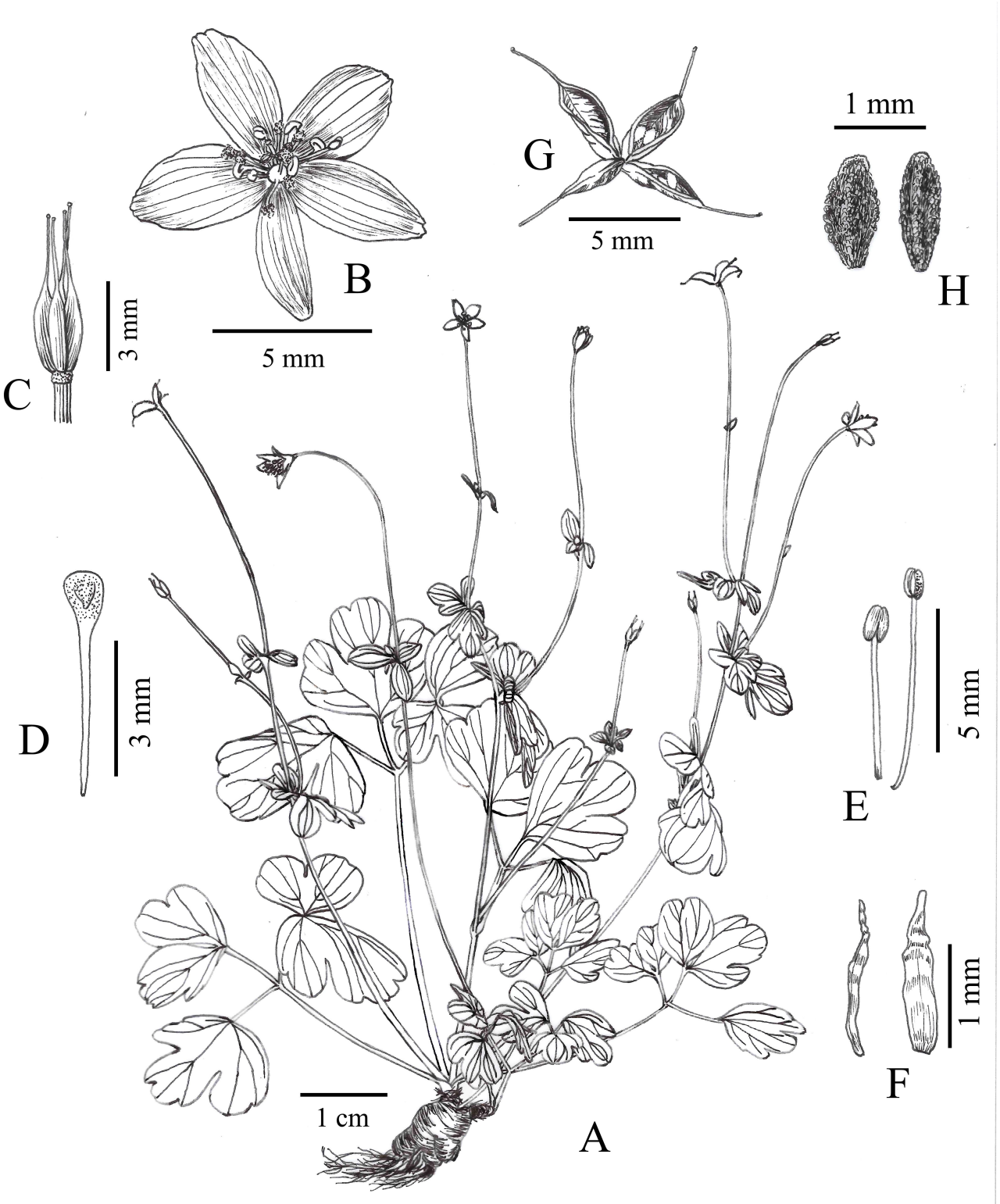

FIGURE 1. *Semiaquilegia danxiashanensis*. A. Habit; B. Flower; C. Pistils; D. Petal; E. Stamens; F. Staminodes; G. Follicles; H. Seeds. Drawn by X.Y. Zeng.

FIGURE 2. Holotype sheet of *Semiaquilegia danxiashanensis*.

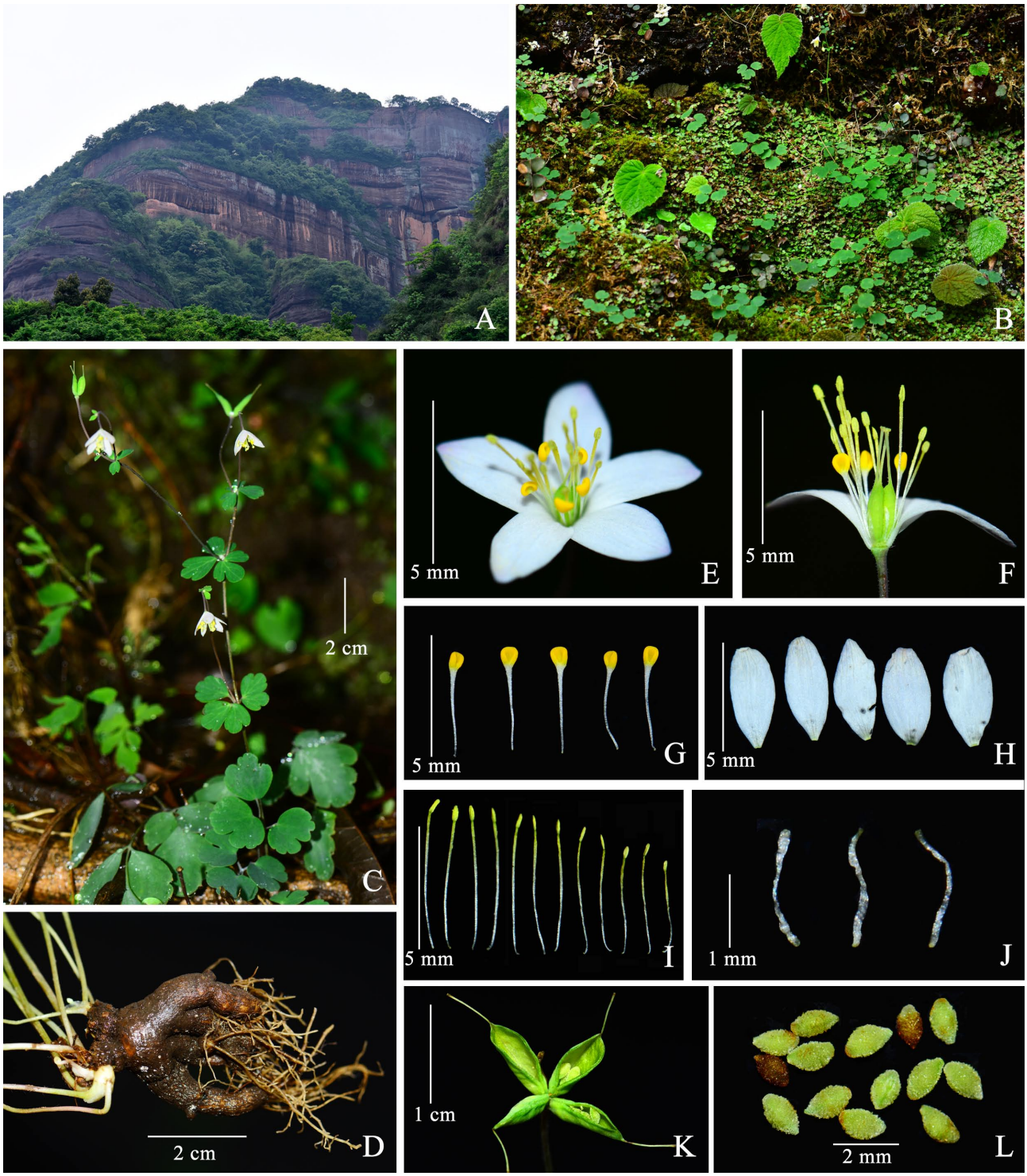

FIGURE 3. *Semiaquilegia danxiashanensis* in the wild (Danxia Shan, Renhua, Guangdong, China). A, B. Habitat; C. Habit; D. Tuber; E. Flower; F. Flower with two sepals removed; G. Petals; H. Sepals; I. Stamens; J. Staminodes; K. Follicles; L. Seeds. Photos by J.J. Zhou and L. Wu.

Materials and methods

Materials examined—Field observations were carried out in Danxiashan National Nature Reserve during February to April in 2018. Morphological characters of *Semiaquilegia danxiashanensis*, particularly those of flowers, were observed and measured in the field. Morphological variation was measured on 30 individuals from the four currently known populations (seven or eight individuals per population) using a ruler and a micrometer. The specimens were

deposited at the Herbarium of Forest Plants in Central South University of Forestry and Technology (CSFI). The conservation status of this new species was evaluated based on the field observations according to the IUCN guidelines (2016).

Molecular methods—We sampled five individuals of *Semiaquilegia danxiashanensis* from the four currently known populations (one or two sampled individuals per population) as well as four samples of *S. adoxoides* (de Candolle 1817: 324) Makino (1902: 119) from Hunan and Guizhou provinces in China for molecular phylogenetic analyses. Total genomic DNA was extracted from silica-gel dried leaves using the CTAB method according to Doyle & Doyle (1987). DNA sequences of the plastid *trnL-F* region and the nuclear ribosomal internal transcribed spacer (ITS) were selected as the markers for molecular phylogenetic study as in a previous study of *Semiaquilegia* (Huang *et al.* 2017). We used the same primers as originally proposed from the previous studies for amplification and sequencing (White *et al.* 1990; Taberlet *et al.* 1991) 'ITS1': AGAAGTCGTAACAAGGTTTCCGTAGG and 'ITS4': TCCTCCGCTTATTGATATGC for the ITS region and 'c': CGAAATCGGTAGACGCTACG and 'f': ATTTGAACTG-GTGACACGAG for the plastid *trnL-F* fragment. PCR conditions were an initial denaturation at 94℃ for 5 min, followed by 35 cycles of 30 sec at 94℃, 30 sec at 52℃, and 1 min at 72℃, with a final extension at 72℃ for 10 min. Subsequent steps were as described in Huang *et al.* (2017). All the samples were successfully amplified and sequenced and a total of 18 sequences (9 ITS sequences and 9 *trnL-F* sequences, respectively) were newly obtained.

We queried the representatives of the newly generated *trnL-F* and ITS sequences in GenBank using BLAST search and found that the most similar sequences were those of *Semiaquilegia adoxoides*, indicating that *S. danxiashanensis* should indeed be a member of *Semiaquilegia.* Referring to Huang *et al.* (2017), we downloaded 26 *trnL-F* sequences and 29 ITS sequences of *Semiaquilegia adoxoides*, *S. guangxiensis* Liu & Huang (2017: 183), and a number of other closely-related genera [*Aquilegia* Linnaeus (1753: 533), *Dichocarpum*, *Enemion* Rafinesque (1820: 70), *Isopyrum* Linnaeus (1753: 557), *Leptopyrum* Reichenbach (1832: 747), *Paraquilegia* Drummond & Hutchinson (1920: 152), *Thalictrum* Linnaeus (1753: 545) and *Urophysa* Ulbrich (1929: 868)] of the Ranunculaceae from GenBank and used together with the newly acquired sequences in the following phylogenetic reconstruction. A total of 35 *trnL-F* and 38 ITS sequences of 16 species from 9 genera were included in the present analysis to test the validity that these samples represent an independent new species and to reveal its phylogenetic position. The most distant outgroup, a clade consisting of *Leptopyrum*, *Isopyrum*, *Paraquilegia* and *Thalictrum*, was used to root the phylogenetic tree as proposed by a previous larger-scale study of the family (Wang & Chen 2007). The GenBank accession numbers of the downloaded sequences are listed in Table 1.

TABLE 1. Information of the taxa included in the molecular phylogenetic analyses. Newly generated sequences are highlighted in bold.

Species	Voucher	Locality	GenBank accession no.	
			trnL-F	ITS
Aquilegia ecalcarata Maxim.	*Wang W. 117*	Chongqing, China	EF437096	AEU75657
A. oxysepala Trautv. & C.A. Mey.	*Chen Z.D. 001, AF01*	Jilin, China	EF437097	JX233769
Dichocarpum dalzielii (J.R. Drumm. & Hutch.) W.T. Wang & P.K. Hsiao	*Wang W. 111*	Guizhou, China	EF437098	EF437115
D. sutchuenense (Franch.) W.T. Wang & P.K. Hsiao	*Wang W. 069*	Guizhou, China	EF437099	EF437116
Enemion raddeanum Regel	*Chen Z.D. 2090*	Jilin, China	EF437100	EF437117
Isopyrum anemonoides Karelin & Kirilow	*Wundish U. 177*	Xinjiang, China	EF437101	EF437118
I. manshuricum Kom.	*Wang W. LN004*	Liaoning, China	EF437102	EF437119
I. thalictroides L.	–	Poland	EF437103	EF437120
Leptopyrum fumarioides (L.) Rchb.	*Man Y.G. T101*	Xinjiang, China	EF437104	EF437121
Paraquilegia microphylla (Royle) J.R. Drumm. & Hutch.	*Li C.Y. 001*	Chongqing, China	EF437105	EF437122
Semiaquilegia adoxoides (DC.) Makino	*Shao Q. 001*	Hunan, China	EF437106	EF437123
S. adoxoides	*Y15042102*	Guilin, Guangxi, China	KY283985	KY283969
	Y15042103	Guilin, Guangxi, China	KY283986	KY283970
	Y15042104	Guilin, Guangxi, China	KY283987	KY283971

...continued on the next page

TABLE 1. (Continued)

Species	Voucher	Locality	GenBank accession no.	
			trnL-F	ITS
	Y15042105	Guilin, Guangxi, China	KY283988	KY283972
	Y15042106	Guilin, Guangxi, China	KY283989	KY283973
	Y15042107	Guilin, Guangxi, China	KY283990	KY283974
	Y15042108	Guilin, Guangxi, China	KY283991	KY283975
	Y15042109	Guilin, Guangxi, China	KY283992	KY283976
	Y15042110	Guilin, Guangxi, China	–	KY283977
	Y13030601	Guilin, Guangxi, China	KY283983	KY283978
	Y13031901	Guilin, Guangxi, China	–	KY283980
	Wu L. 6353	Changsha, Hunan, China	–	–
	Tu R.H. 01	Wugang, Hunan, China	–	–
	Zhang Q.	Zunyi, Guizhou, China	–	–
	Zhang Q.	Tongren, Guizhou, China	–	–
S. *guangxiensis* Yan Liu & Y.S. Huang	*Y13030901*	Guilin, Guangxi, China	KY283994	KY283981
	Y14041401	Guilin, Guangxi, China	KY283995	KY283982
	Y15042101	Guilin, Guangxi, China	–	KY283983
S. danxiashanensis L. Wu, J.J. Zhou, Q. Zhang & W.S. Deng	***DXTK001***	Shaoguan, Guangdong, China	–	–
	DXTK002	Shaoguan, Guangdong, China	–	–
	DXTK003	Shaoguan, Guangdong, China	–	–
	DXTK004	Shaoguan, Guangdong, China	–	–
	DXTK031201	Shaoguan, Guangdong, China	–	–
Thalictrum robustum Maxim.	*Wang W. 038*	Guizhou, China	EF437108	EF437125
T. javanicum Blume	*Wang W. 067*	Guizhou, China	EF437107	EF437124
Urophysa henryi (Oliv.) Ulbr.	*Wang W. 096*	Guizhou, China	EF437109	EF437126

DNA sequences were aligned using the program MUSCLE 3.8.31 (Edgar 2004) and adjusted manually in Bioedit 5.0.9 (Hall 1999). A few sites which were ambiguously aligned even after the manual adjustment were pruned before phylogenetic analyses. We reconstructed the phylogeny using maximum likelihood (ML) and Bayesian inference (BI). First, we reconstructed the maximum likelihood trees based on the ITS and *trnL-F* data, respectively, and compared the ML trees to check whether any phylogenetic conflict existed between the plastid and nuclear data. The phylogenetic relationships were essentially congruent between the plastid and ITS ML trees, with slight topological differences outside of *Semiaquilegia* with weak support, therefore, we reconstructed phylogeny based on the concatenated data to further test the affinity of the new species. ML analyses were performed using RAxML-VI-HPC (Stamatakis 2006) with the substitution model GTR+G and 1000 rapid bootstrap searches (BS). Bayesian analyses were conducted in

MrBayes 3.2.6 (Ronquist *et al.* 2012) with the optimal substitution model K81uf+I+G selected by ModelTest (Posada & Crandall 1998) according to the Akaike Information Criterion (AIC). All BI analyses were run for 100,000,000 generations with four chains in two parallel runs and sampled every 5000 generations with a burn-in of the first 5000 trees. The convergence of the two parallel runs was determined by a splitting frequency less than 0.005. All other parameters were set as default.

Results and discussion

Our observations of herbarium specimens and living plants indicate that *Semiaquilegia danxiashanensis* is indeed similar to species of *Dichocarpum* in having clawed petals golden-yellow in the upper part and much smaller than the sepals. It is also similar to species of *Semiaquilegia* in the presence of staminodes (ca. 5) and in usually having four pistils and ellipsoid and densely rugose seeds (Figs. 3–5; Table 2) . Our molecular analyses of both the nuclear ITS and the plastid *trnL-F* region have strongly confirmed *S. danxiashanensis* as a member of *Semiaquilegia*.

FIGURE 4. *Semiaquilegia guangxiensis* in the wild (Jiangyue village, Yongfu, Guangxi, China). A. Habitat; B. Habit; C. Flowers; D. Sepals; E. Petals; F. Follicles. Photos by Y.S. Huang and Y. Liu.

TABLE 2. Morphological comparisons of Semiquilegia species.

Character	*S. adoxoides*	*S. danxiashanensis*	*S. guangxiensis*	*S. quelpaertensis*
Tuber	thin, 1–2 cm long, 0.3–0.5 mm in diam.	thick, to 3 cm long, ca. 2 cm in diam.	thick, 2–5 cm long, ca 1.5 cm in diam.	thick, 3–5 cm long, 0.6–1 cm in diam.
Basal leaf	ovate, suborbicular, or reniform, 1.2–3 × 1.2–4.5 cm	broadly ovate to triangular-ovate, 3–5 × 3–8.5 cm	suborbicular, or ovate to triangular-ovate, 3–9.5 × 3–9.5 cm	suborbicular, or ovate to triangular-ovate, 2–3.5 ×2–3.5 cm
Flower	4–6 mm in diam.	7–9 mm in diam.	15–25 mm	8–10 mm
Pedicel	1–2.5 cm long	2.0–12 cm long	2.5–12 cm long	0.8–2.5 cm long
Sepal	narrowly elliptic, 4–6 × 1.2–2.5 mm	elliptic or obovate, 5.2–8.3 × 2–2.5 mm	broadly elliptic or obovate, 10–20 × 5–10 mm	narrowly elliptic, 7–8 × 3.0–3.5 mm
Petal	spatulate, 2.5–3.5 mm long, base cystic	clawed, limbs suborbicular, ca. 1 mm long, golden-yellow, retuse, claws obviously longer than limbs, ca. 3.5 mm long.	spatulate, 4–6 mm long, yellow; base tubulose	spatulate, 3.0–3.5 mm long, yellow, base cystic
Stamen	8–14	12–16	20–30	16–22;
Staminode	ca. 2, as long as filaments	ca. 5, 1/3–1/2 as long as filaments	ca. 10, half the length of the filaments	(4–)6, 1/2–2/3 as long as filaments
Follicle	6–7 mm long, ca. 2 mm in diam.	6–7 mm long, ca. 2.5 mm in diam.	ca. 1 cm long, ca. 3 mm in diam.	7–9 mm long, ca. 3 mm in diam.
Seed	obovoid, 1.5 mm long, rugose on surface	ellipsoid, ca. 1.5 mm long, rugose on surface	ellipsoid, 1.5–2.5 mm long, rugose on surface	obovoid, 1.5–2.0 mm long, rugose on surface

The combined matrix used for phylogenetic reconstruction had a length of 1503 characters (*trnL-F*: 901 characters, ITS: 602 characters) including 178 parsimony informative sites (*trnL-F*: 76 characters, ITS: 102 characters), 270 variable but parsimony uninformative sites (*trnL-F*: 122 characters, ITS: 148 characters) and 1233 constant sites (*trnL-F*: 779 characters, ITS: 454 characters). The consistency index (CI), retention index (RI) and homoplasy index (HI) were 0.827, 0.917 and 0.173 respectively for the combined data (0.760, 0.863, and 0.240, and 0.943, 0.979 and 0.057 for the ITS and *trnL-F* data, respectively).

Both methods of maximum likelihood and Bayesian inference based on the *trnL-F*, ITS and concatenated data generated essentially congruent phylogenetic relationships except minor topological differences outside of *Semiaquilegia* with weak support (Figs. 6–8). Therefore we present the most resolved topology generated from BI analysis based on the combined data (Fig. 8). The analysis in the present study yielded generally consistent intergeneric relationships with those obtained in a previous study by Wang & Chen (2007), and both studies indicated that the three *Isopyrum* species are separated on two distant branches, supporting the suggested resurrection of *Paropyrum* Ulbrich (1925: 218) to accommodate *I. anemonoides* Karelin & Kirilow (1842: 135) (Wang & Chen, 2007). The discrepancy is that *Semiaquilegia* is resolved as the sister to *Urophysa* in our study (BS = 61; PP = 0.65), while it was suggested to be closest to *Aquilegia* (BS = 71; PP = 0.77) in the study by Wang & Chen (2007), but both with weak supports.

In *Semiaquilegia*, it showed that all of the three sampled species, namely *S. guangxiensis*, *S. adoxoides* and *S. danxiashanensis*, are clustered together as a monophyletic group (BS = 99%; PP = 1.00), with the latter two species forming sister taxa (BS = 100%; PP = 1.00). Within *Semiaquilegia*, all sampled accessions of each of these three species are clustered as monophyletic groups, respectively, indicating genetic isolation among the three species (BS = 97%, 100% and 100%; PP = 1.00, 1.00 and 1.00 for the accessions of *S. adoxoides*, *S. danxiashanensis* and *S. guangxiensis*, respectively). This genetic distinction in addition to the marked morphological differences justifies that the populations we named as *S. danxiashanensis* should represent an independent new species.

Taxonomic treatment

Semiaquilegia danxiashanensis L. Wu, J.J. Zhou, Q. Zhang & W.S. Deng, ***sp. nov.*** (Figs. 1–3)

Type:—CHINA. Guangdong province: Renhua county, Danxiashan National Nature Reserve, alt. 122 m, wet cliffs, 25°1′N, 113°44′E, 17 March 2018, *J.J. Zhou & W.S. Deng DXTK001* (holotype CSFI 063660, isotypes CSFI, IBK).

Diagnosis:—*Semiaquilegia danxiashanensis* is distinguished in the genus by having clawed petals with golden-yellow suborbicular limbs and claws obviously longer than limbs.

Description:—Perennial herbs. Tuber thick, blackish brown, to 3 cm long, ca. 2 cm in diam. Stems 1–6, 10–30 cm in height, branches with spreading white hairs. Basal leaves numerous, ternately pinnate; petiole 2–11 cm long, sparsely spreading white pubescent, basal petiole sheathed; leaf blade broadly ovate to triangular-ovate, 3–5 × 3–8.5 cm; leaflets flabellate-rhombic to obovate-rounded, 1.0–2.7 × 1.0–3.5 cm, 3-parted; segments unequally lobed, adaxially green, abaxially pale green, both surfaces glabrous, or sometimes pubescent abaxially along nerves; petioles of the middle leaflets a little longer than those of the lateral ones, 1–3.5 cm long, sparsely pubescent . Cauline leaves subsessile, leaf blades similar to those of basal leaves but smaller. Inflorescences monochasial, 1- or 2-flowered; bracts oblanceolate to obovate, entire or 3-lobed. Flowers 7–9 mm in diam. Pedicel 2.0–12 cm long, sparsely pubescent. Sepals white, rarely pink, elliptic or obovate, 5.2–8.3 × 2–2.5 mm, apex rounded or obtuse, usually emarginated. Petal limbs suborbicular, ca. 1 mm long, golden-yellow, retuse, glabrous; claw obviously longer than limb, ca. 3.5 mm long. Stamens 12–16; anthers ca. 0.6 mm long; filaments unequal in length, 3.5–4.5 mm long; staminodes ca. 5, linear-lanceolate, white, membranous, glabrous, ca. 1.5 mm long. Pistils 2–4, sparsely pubescent. Follicles ovate-oblong, 6–7 mm long, ca. 2.5 mm in diam., striae transversely raised; style persistent, straight, filiform when drying, 3–4.5 mm long, glabrous. Seeds ellipsoid , ca. 1.5 mm long, surface rugose.

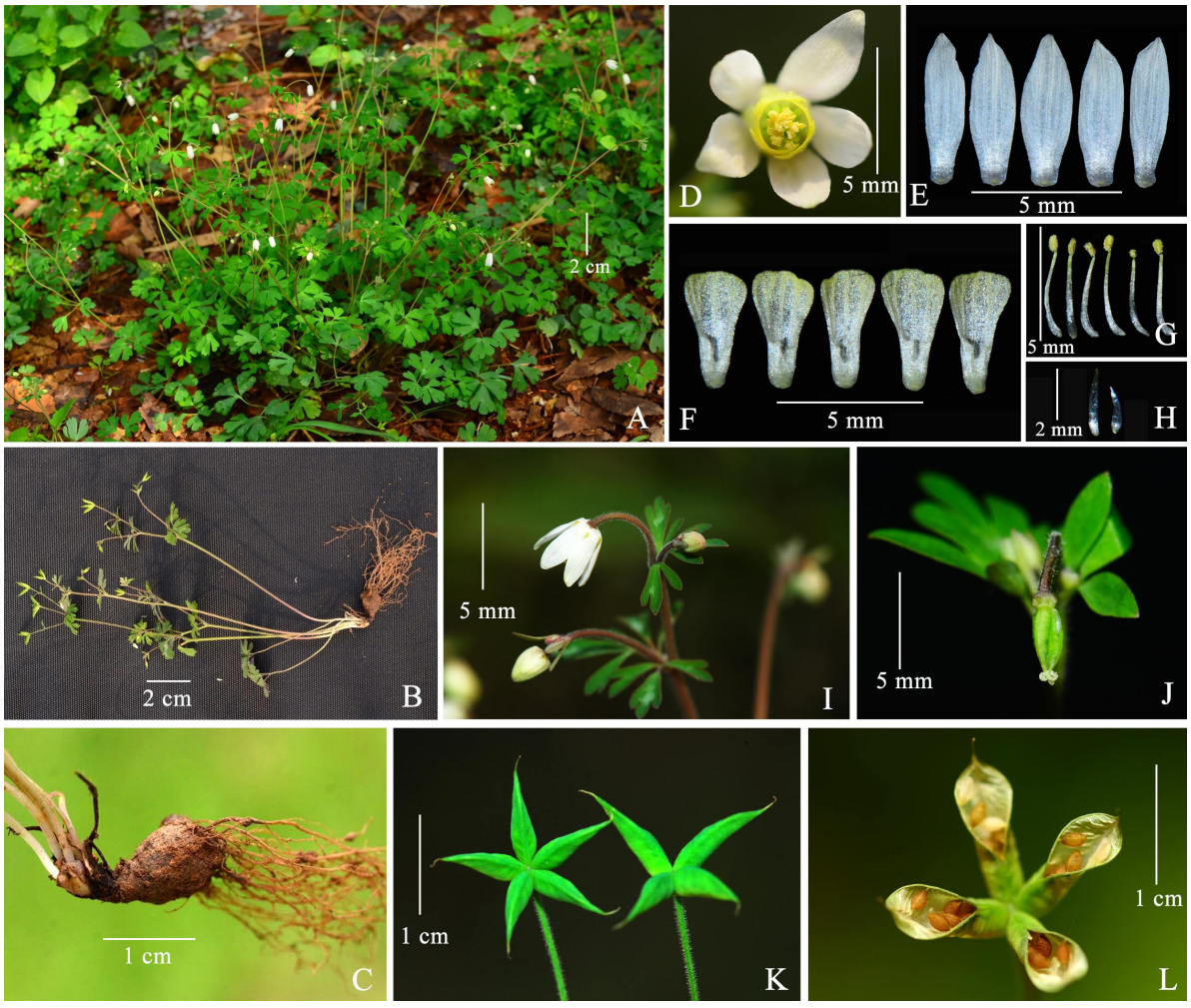

FIGURE 5. *Semiaquilegia adoxoides* in the wild (Yuelu Mountain, Changsha, China). A, B. Habit; C. Tuber; D. Flower; E. Sepals; F. Petals; G. Stamens; H. Staminodes; I. Inflorescence; J. Pistils; K. Follicles (immature); L. Follicles (mature). Photos by J.J. Zhou and L. Wu).

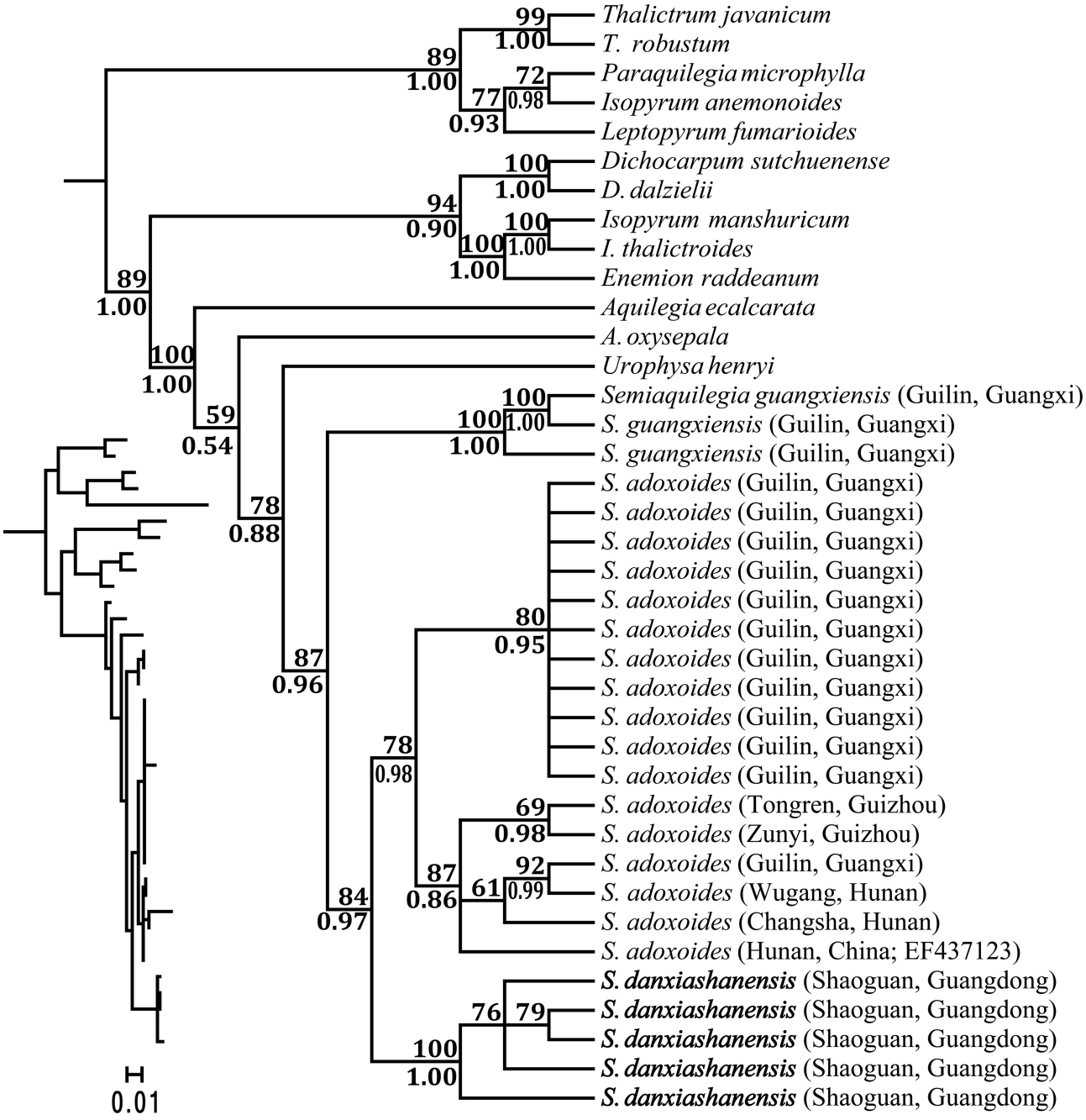

FIGURE 6. The ITS maximum likelihood phylogenetic trees with BI posterior probability/ML bootstrap support values (>0.5 or 50%) shown below and above the branch around the corresponding node. The accessions of *Semiaquilegia danxiashanensis* are highlighted in bold.

Phenology:—Flowers from February to April; fruits from March to May.

Etymology:—The specific epithet "*danxiashanensis*" is derived from the type locality of the new species, i.e. Danxia Shan in Renhua county, Guangdong, China.

Distribution and habitat:—*Semiaquilegia danxiashanensis* is currently known only from the type locality. The plants grow on wet cliffs. We have carried out several expeditions and investigations in the surrounding places with non-Danxia landform habitats but did not find this species there. Therefore, we presume that this new species may be endemic to Danxia Shan, as some other taxa, e.g. *Firmiana danxiaensis* (Chen *et al.* 2015).

Conservation status:—Four populations of *Semiaquilegia danxiashanensis* with approximately 300 mature individuals at each site are currently known, and all are from Danxiashan National Nature Reserve in northern Guangdong, southern China. Danxiashan National Nature Reserve was built in 1995 and the main purpose of the

establishment is to protect its unique landform, so that the forests in the reserve and the habitats of this new species have been under continuous and effective protection as well. Taken together, the species is assigned to the status of 'Near Threatened' (NT) following the guidelines of IUCN (2016).

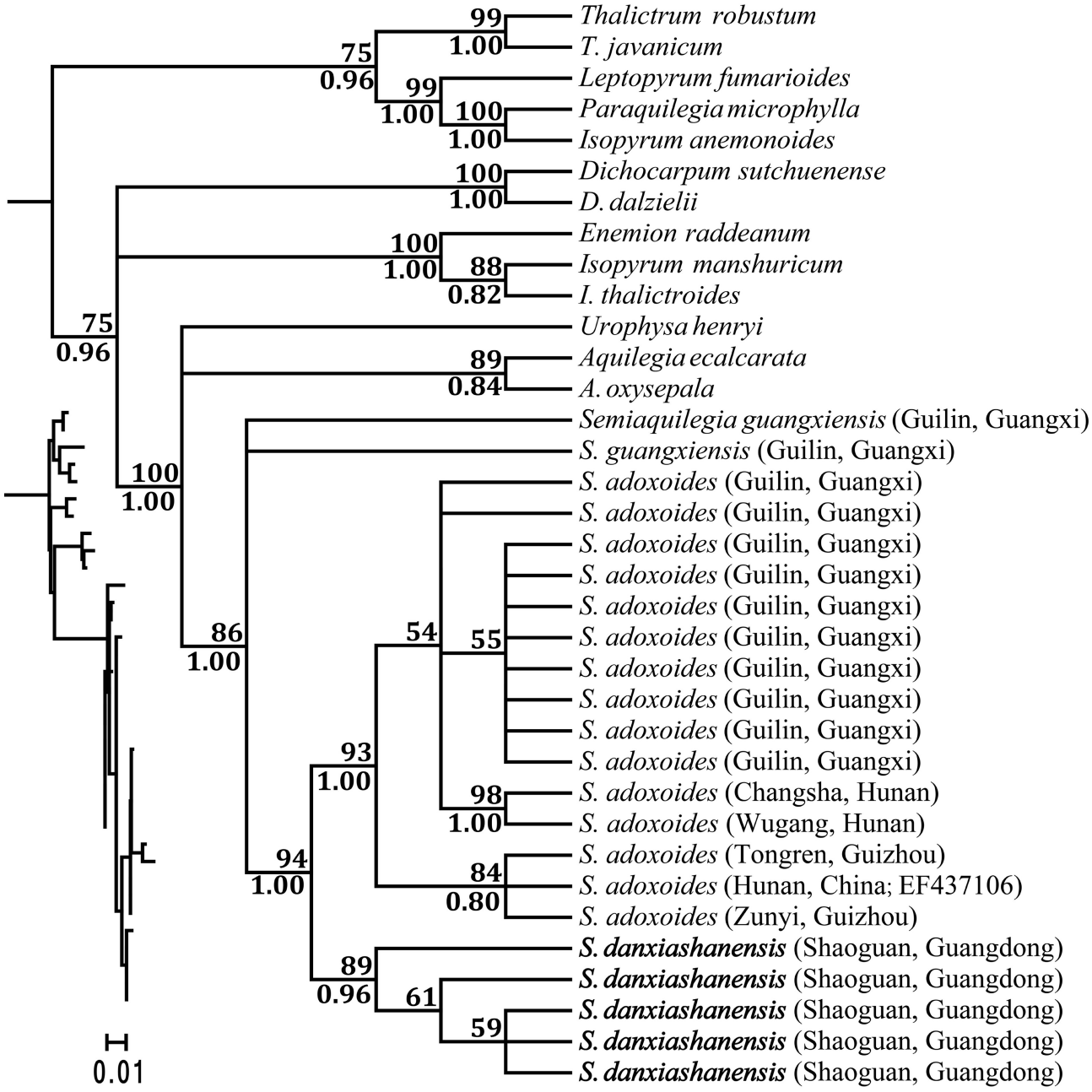

FIGURE 7. The *trnL-F* maximum likelihood phylogenetic trees with BI posterior probability/ML bootstrap support values (>0.5 or 50%) shown below and above the branch around the corresponding node. The accessions of *Semiaquilegia danxiashanensis* are highlighted in bold.

Additional specimen examined (paratypes):—China. Guangdong: Renhua county, Danxia Shan, 12 March 2018, *J. J. Zhou & L. Wu DXTK031201* (CSFI), 17 March 2018, *J. J. Zhou & W. S. Deng DXTK002* (CSFI, IBK), *J. J. Zhou & W. S. Deng DXTK003* (CSFI), *J. J. Zhou & W. S. Deng DXTK004* (CSFI), *L. Wu 7011* (CSFI).

Notes:—*Semiaquilegia danxiashanensis* is a distinctive species in the genus by having clawed petals with suborbicular limbs, a character otherwise mainly occurring in the genus *Dichocarpum*. Indeed, the discovery of this new species has largely obscured the morphological distinction of *Dichocarpum*.

The genus *Semiaquilegia* consists of four species with the description of *S. danxiashanensis* (Table 2). They can be keyed out as follows.

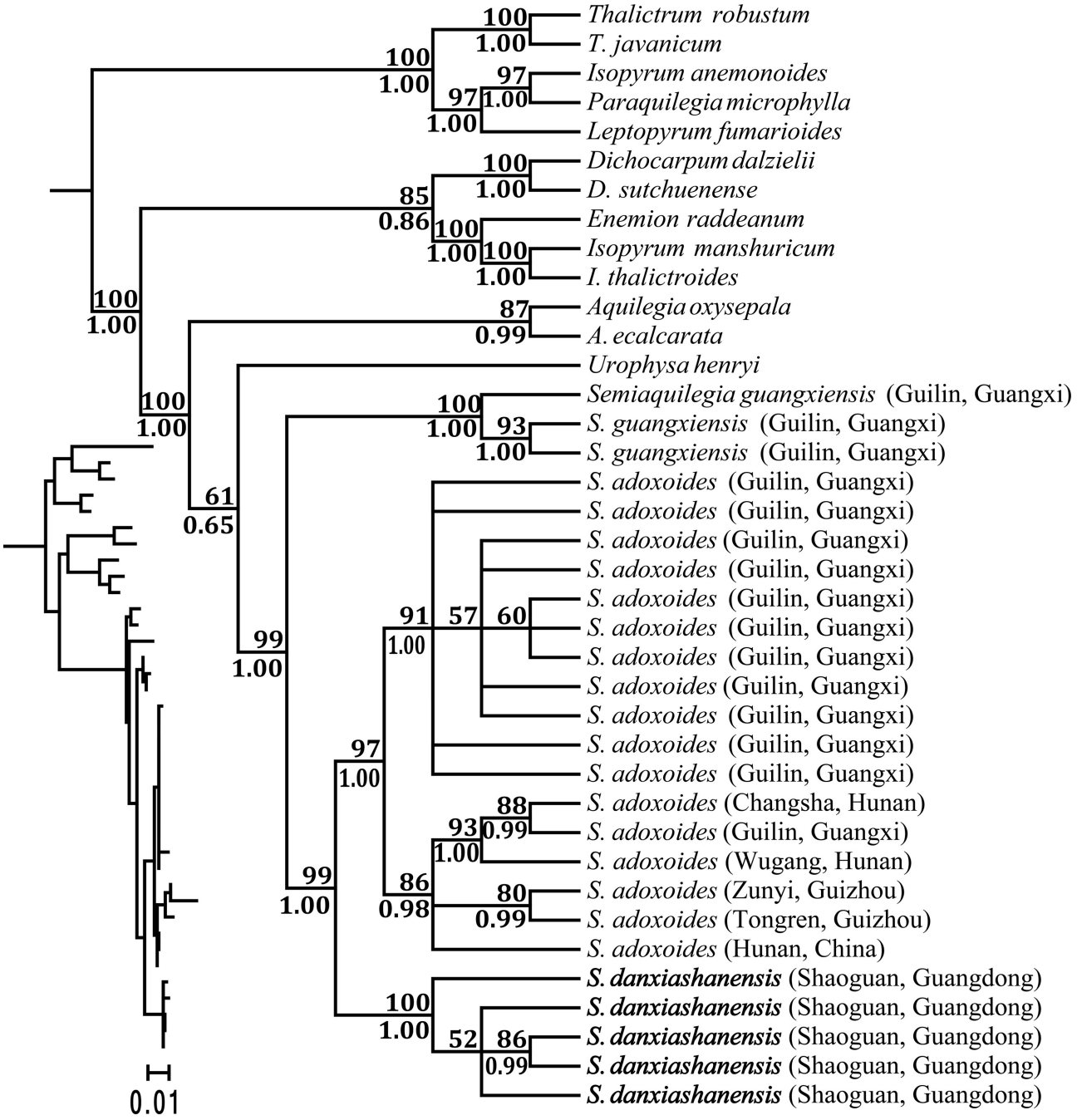

FIGURE 8. The Bayesian phylogenetic trees from the analyses of the combined data of the ITS and chloroplast *trnL-F* regions. BI posterior probability/ML bootstrap support values (>0.5 or 50%) are shown below and above the branch respectively around the corresponding node. The accessions of *Semiaquilegia danxiashanensis* are highlighted in bold.

Key to species of *Semiaquilegia*

1. Petals clawed, limbs suborbicular, ca. 1 mm long, claws ca. 3.5 mm long; fruit with a 3–4.5 mm long and filiform persistent style..... *S. danxiashanensis*
- Petals spatulate, 2.5–3.5 mm long, without claw; fruits without or with a less than 2 mm long persistent and filiform style.....2
2. Flowers 15–25 mm in diam.; sepals 10–20 mm long.....*S. guangxiensis*
- Flowers usually 4–10 mm in diam.; sepals shorter than 9 mm 3
3. Flowers 4–6 mm in diam.; staminodes ca. 2 ..... *S. adoxoides*
- Flowers 8–10 mm in diam.; staminodes 4–6..... *S. quelpaertensis*

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