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Research

Two new species of *Litostigma* (Gesneriaceae) from the China–Vietnam border area based on morphological and molecular data, adding new stigma characters for the genus

You-Sheng Chen, Yun-Feng Huang, Lian-Sheng Xu and Bing-Mou Wang

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Litostigma is a recently described small genus with two species confined to limestone karsts in southwestern China and adjacent Vietnam. During the course of field works on the limestone karsts of the China–Vietnam border area, two unknown species of *Litostigma* were collected. Molecular phylogenetic analyses based on nuclear ITS sequences strongly confirm the placements of the two new species in *Litostigma*. However, these two new species can be distinguished from the previously known species by distinct morphological characters. Thus, *Litostigma pingbianense* and *L. napoense* are recognized as two new species. This adds to the morphological variability of the genus. Most importantly, both simple and divided stigmas does occur in this genus.

Keywords: African violet family, ITS phylogeny, *Litostigma napoense*, *Litostigma pingbianense*

Introduction

Litostigma Wei et al. is a small genus in Gesneriaceae with only two species first described from from Guizhou and Yunnan, China (Wei et al. 2010). These two species are both narrowly distributed and confined to limestone karsts. Recently, Lu et al. (2020) reported *L. crystallinum* from northern Vietnam, very close to the Sino-Vietnamese border. The genus is characterised by its large flowers in comparison to its small leaves, slightly revolute leaf margins, 1-flowered cymes, crateriform or disciform stigma, and long ovoid capsule (Wei 2010, Wei et al. 2010).

The Sino-Vietnamese limestone karsts are renowned for their spectacular landscape and rich biodiversity (Myers et al. 2000, Clements et al. 2006). Interestingly, many genera and a large number of species of Gesneriaceae are endemic to this area (Wei 2010, Guo et al. 2015). In fact, new species from this area are constantly discovered and described (Xu et al. 2017, Hong et al. 2018, Li et al. 2019, Chen et al. 2020a, Qin et al. 2020, Xin et al. 2020).



During a recent extensive floristic survey in Pingbian county of Yunnan province and Napo county in Guangxi Zhuangzu Autonomous Region of China, we collected two unknown Gesneriaceae plants. Both of them grow on the rocks at the entrance of a limestone cave. We made a preliminary judgment that these two species belong to *Litostigma*, but they did not conform to the characteristics of the two known species of that genus. Further molecular studies (nuclear ITS sequences) and morphological comparisons indicated that these two plants represented two undescribed species of *Litostigma*.

Material and methods

Taxon sampling and DNA sequencing

A total of 50 accessions were included in this study, including 4 species (4 samples) of *Litostigma* and 46 related species (46 samples) referring to Wei et al. (2010) (Table 1, Supporting information). The sequences of *Litostigma* were newly acquired here (Table 1). DNA was extracted from leaves dried over silica-gel using the CTAB Plant Genomic DNA Extraction kit (Biomed Beijing). The nuclear ITS region were then amplified as described by Yuan et al. (2015) and improved. The 25 µl volume polymerase chain reactions (PCRs) contained 20 ng of plant DNA, 200 µM dNTPs, 0.25 µM primer, 1 U Taq polymerase, 1× Taq buffer (Takara Biotechnology Co., Dalian, China). The PCR amplification profiles were identical for the three fragments: one cycle at 94°C for 4 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1.5 min; and one cycle at 72°C for 10 min. The PCR products were purified using Magnetic Beads DNA Gel Extraction Kit (Ensuer Biologicals, Shanghai) and sequenced using an ABI 3130X sequencer in Shanghai Majorbio Biopharm Technology Co., Ltd.

Phylogenetic analysis

The obtained sequences were first assembled in ContigExpress (Informax, Bethesda, MD, USA). Phylogenetic analysis was then performed in PhyloSuite ver. 1.2.1 (Zhang et al. 2020). We aligned the sequences with MAFFT ver. 7.313 (Katoh and Standley 2013) using the ‘--auto’ strategy and normal alignment mode and then adjusted it manually in BioEdit 7.0.9

Table 1. Sample information for newly acquired ITS sequences.

Taxon	Origin	Voucher
<i>Litostigma coriaceifolium</i>	China, Guizhou, Xingyi county	W. G. Wei & F. Wen 0701 (IBK)
<i>Litostigma crystallinum</i>	China, Yunnan, Malipo county	Y. M. Shui et al. 53856 (KUN)
<i>Litostigma napoense</i> sp. nov.	China, Guangxi, Napo county	Y. F. Huang & Y. Nong 79975 (GXMI)
<i>Litostigma pingbianense</i> sp. nov.	China, Yunnan, Pingbian county	Y. S. Chen et al. PB0718 (IBSC)

(Hall 1999). The best partitioning scheme and evolutionary models were selected using PartitionFinder2 (Lanfear et al. 2016), with rcluster algorithm and AICc criterion. Three species of *Corallodiscus* Batalin were chosen as outgroups in the following phylogenetic analysis, based on the results of Wei et al. (2010). Maximum likelihood (ML) phylogenies were inferred using IQ-TREE (Nguyen et al. 2015) under the Edge-linked partition model for 10 000 standard bootstraps. Bayesian inference (BI) phylogenies were inferred using MrBayes 3.2.6 (Ronquist et al. 2012) under the N/A model (2 parallel runs, 2 000 000 generations), in which the initial 25% of the sampled data were discarded as burn-in. The Bayesian posterior probability (PP) from BI analysis and bootstrap values (BS) from ML of each branch was obtained. Nodes with PP ≥ 0.95 (Ronquist and Huelsenbeck 2003) and BS ≥ 75% (Hillis and Bull 1993) were considered well supported.

Results and discussion

The BI tree (Fig. 1) and ML tree (Supporting information) had inconsistent topologies. However, in both trees the two new species and the two previously known species of *Litostigma* form a well-supported clade (PP = 1, BS = 100%), suggesting that the two new species belong to *Litostigma*. *Litostigma pingbianense* is related to *L. coriaceifolium*, which is consistent with the results of molecular phylogeny (PP = 1, BS = 99%). *Litostigma napoense* is the basal group of *Litostigma*, sister to all the other species of *Litostigma* (PP = 1, BS = 99%). Interestingly, *L. napoense* has a different stigma feature than the other species of the genus. The discovery of *L. napoense* thus modify morphological circumscription of *Litostigma*. Previously, one important feature defining *Litostigma* was the crateriform or disciform simple stigma (Wei et al. 2010). But now, *Litostigma* has both simple and divided stigma. Both simple and divided stigmas occur in the same Gesneriaceae genera, e.g. in *Chiritopsis* W. T. Wang, *Primulina* Hance and *Tremacron* Craib (Wei 2010, Li 2019).

Conclusions

Taxonomic treatment

***Litostigma pingbianense* Y. S. Chen & B. M. Wang, sp. nov. (Fig. 2,3)**

Type: China, Yunnan, Pingbian county, Watan, Wujiazhai, at entrance of a limestone cave, 900 m a.s.l., 8 Apr 2020, Y. S. Chen et al. PB0718 (holotype: IBSC; isotypes: IBSC).

Etymology

The epithet of the new species refers to its type locality, Pingbian county Yunnan Province of China. Chinese name: Ping Bian Ao Zhu Ju Tai (屏边凹柱苣苔).

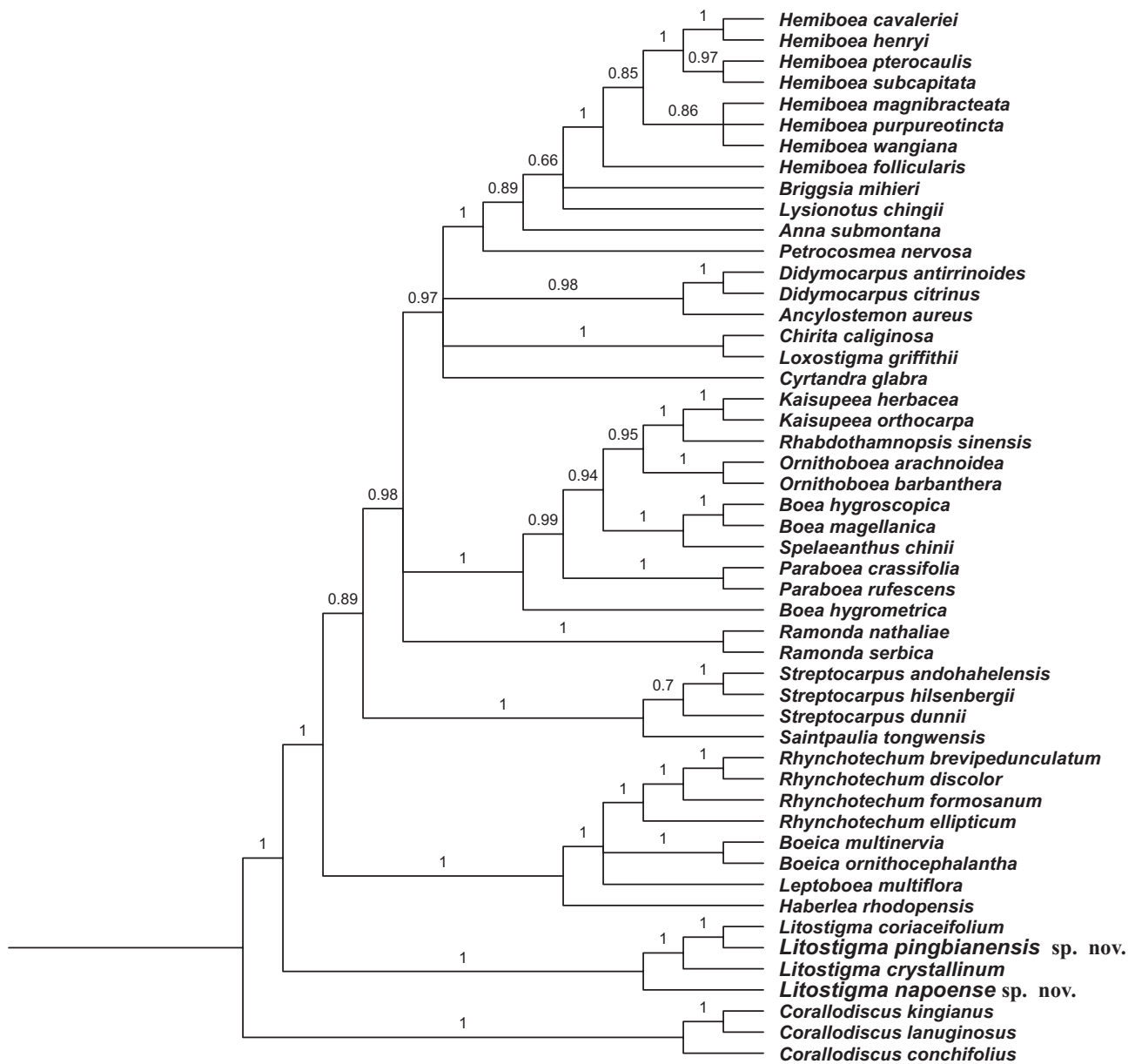


Figure 1. BI tree inferred from ITS sequences. Numbers above branches are Bayesian posterior probabilities (PP).

Description

Perennial stemless herb. Rhizome branched or unbranched, 1.0–1.5 cm long, 1–2 mm in diameter. Leaves 10–25, all basal; leaf blade coriaceous, elliptic to ovate, 1.5–4.0 cm long, 0.7–1.9 cm wide, rounded or obtuse at apex, cuneiform at base, with margin entire, slightly revolute, glabrous, green above, greyish green beneath; lateral nerves 3–4, distinctly convex beneath, inconspicuous at adaxial surface; petioles 1.5–9.0 cm long, glabrous. Cymes 1–5, 1-flowered; peduncle 2.5–5.0 cm long, glabrous; 2-bracteate, small, linear, ca 3 mm long, ca 0.5 mm wide. Sepals 5, lanceolate, ca 3 mm long, ca 1 mm wide, slightly pubescent outside, glabrous inside, acute at apex. Corolla bluish-purple, infundibuliform, 1.6–2.4 cm long, glabrous; corolla tube with undilated proximal part

1.2–1.5 cm long and dilated distal part 5.0–6.0 mm long, 1.5–1.7 cm in diameter at the orifice, 1.8–2.0 mm in diameter near the base; adaxial lip 3.5–4.0 mm long, 2-parted nearly to the base; abaxial lip 5.0–6.0 mm long, 3-parted nearly to the base, with lobes all orbicular-ovate. Stamens 2 (in anterior position), inserted at ca 5 mm from the corolla tube base; filaments linear, arcuate upward to the adaxial lip, 5.0–6.0 mm long; anthers elliptic, ca 1.5 mm long, prominently villous, coherent adaxially. Staminodes 3, glabrous, inserted at ca 5 mm from corolla tube base, ca 2 mm long. Disc annular, ca 1 mm high. Ovary ovoid-ellipsoid, ca 2 mm long, ca 1 mm in diameter, narrowly ovoid; style pubescent, 11.0–14.0 mm long. Ovary 1-loculed; placentae 2, parietal, intrusive, bifid. Stigma crateriform, ca 1 mm in diameter.

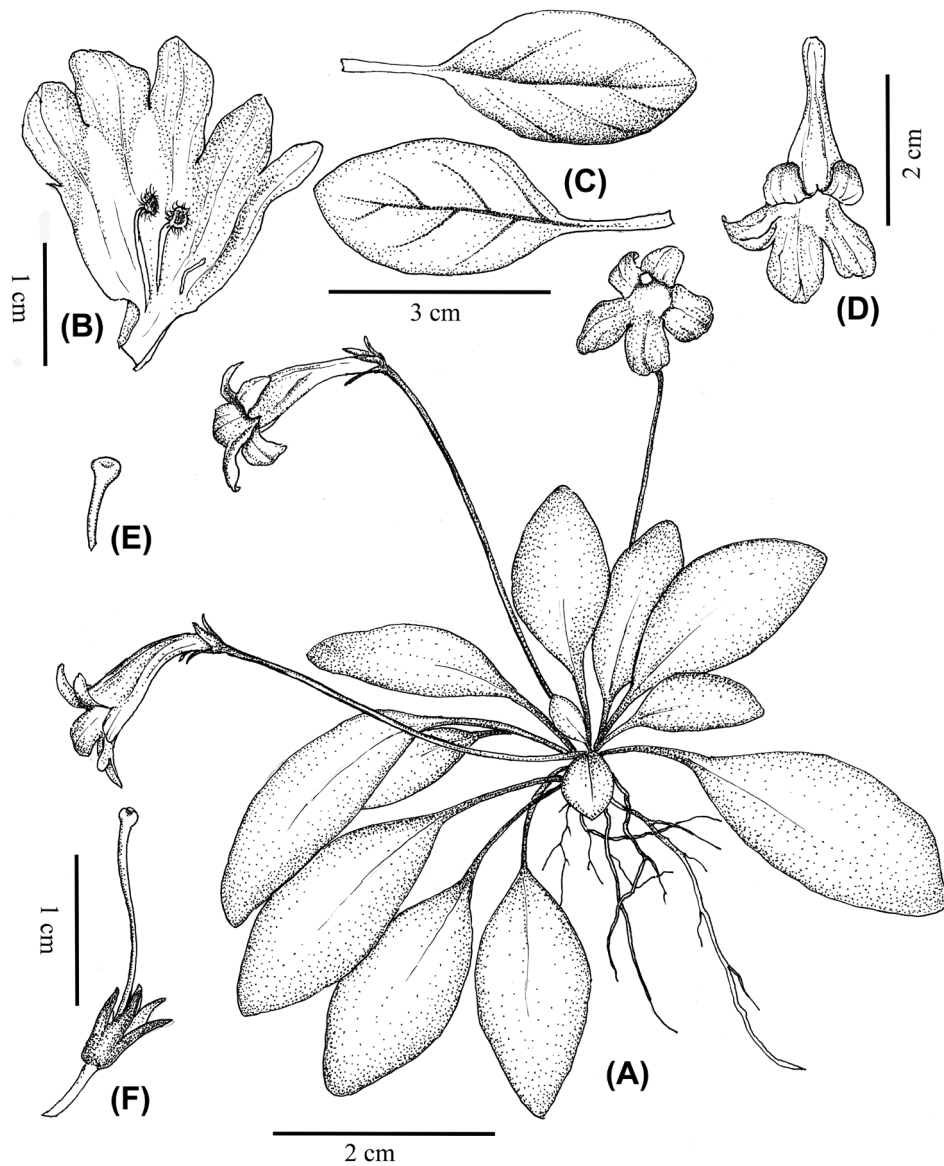


Figure 2. *Litostigma pingbianense* sp. nov. (A) Habit, (B) corolla open with stamens and staminodes, (C) leaf blade, (D) corolla, (E) stigma, (F) calyx and pistil. (Drawn by Bing-Mou Wang based on Y. S. Chen et al. PB0718).

Fruit 3.0–4.0 mm long, narrowly ovoid, pubescent, dehiscent into 4 valves.

Phenology

Flowering from March to April, fruiting from April to June.

Distribution and habitat

Litostigma pingbianense is presently only found as one population in Pingbian county, Yunnan province of China. It grows on the cliff at the entrance of a limestone cave at elevation of ca 900 m.

Conservation status

Litostigma pingbianense is presently known only from one population with no more than 100 individuals. This species may easily become threatened by habitat loss. Due to the small size of the population, it should be considered

critically endangered (CR) based on the IUCN red list criteria (IUCN 2019).

Similar species and phylogenetic position

The results of molecular systematics are consistent with those of morphology: *Litostigma pingbianense* is closest to *L. coriaceifolium*. *Litostigma pingbianense* is mostly similar to *L. coriaceifolium* Y. G. Wei et al. (2010, p. 179) (Fig. 4E–H) in its habit, shape and texture of leaves, shape and color of corolla, and anthers prominently villous, but differs by its leaf blade usually larger (1.5–4.0 cm long, 0.7–1.9 cm wide versus 1.0–2.1 cm long, 0.6–1.1 cm wide), petioles 1.5–9.0 cm long, glabrous (versus 0.4–2.5 cm long, sparsely pubescent), peduncles 2.5–5.0 cm long, glabrous (versus 1.5–2.0 cm long, pubescent), corolla 1.6–2.4 cm long, with two white lines in the throat, glabrous outside (versus 1.6–2.4 cm long, with two

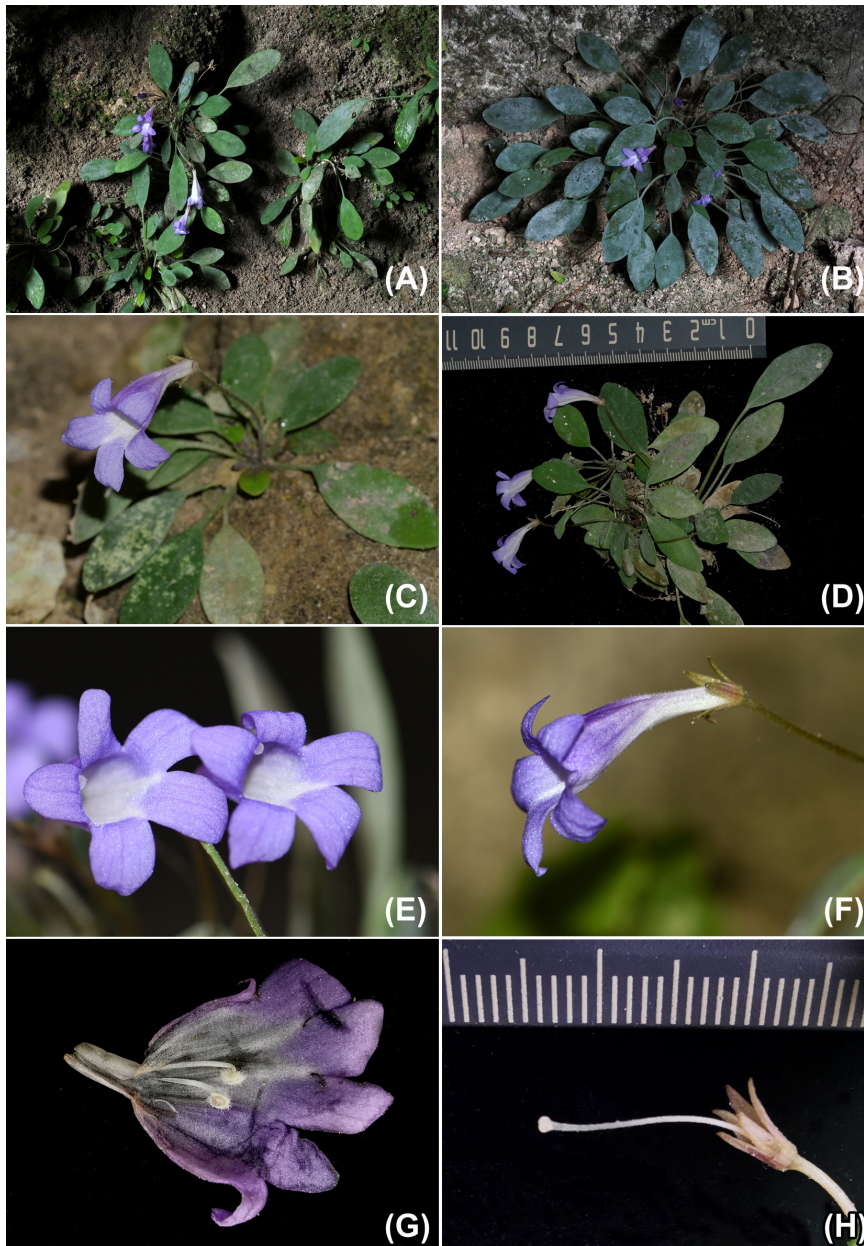


Figure 3. Living plants of *Litostigma pingbianense* sp. nov. (A–D) Habit, (E) corolla in front view, (F) corolla in lateral view, (G) corolla open showing stamens and staminodes, (H) calyx and pistil. (Photos by You-sheng Chen based on Y. S. Chen et al. PB0718).

yellow lines in the throat, pubescent outside), style ca 12 mm long (versus ca 9.5 mm long), filaments arcuate upward to the adaxial lip, 5–6 mm long (versus erect, 3–4 mm long) and fruit 3–4 mm long, pubescent (versus 5–6 mm long, glabrous).

***Litostigma napoense* Y. Feng Huang, B. M. Wang & Y. S. Chen, sp. nov. (Fig. 5, 6)**

Type: China, Guangxi, Napo county, Naidu town, Pojin cun, Jinsanjiao, at entrance of a limestone cave, 650 m a.s.l., 8 Dec 2019, Y. F. Huang & Y. Nong 79975 (holotype GXMI; isotypes GXMI, IBSC).

Etymology

The epithet of the new species refers to its type locality, Napo county, Guangxi Zhuangzu Autonomous Region of China. Chinese name: Na Po Ao Zhu Ju Tai (那坡凹柱苣苔).

Description

Perennial stemless herb. Rhizome subterete, 4–6 mm long, 2–3 mm in diameter. Leaves 15–30, all basal; leaf blade thick papery, elliptic, 3–9 cm long, 1.7–4.3 cm wide, acute to obtuse at apex, broadly cuneiform at base, with margin entire, slightly revolute, glabrous, green above, greyish green beneath; lateral nerves distinct, 4–6 on each side,

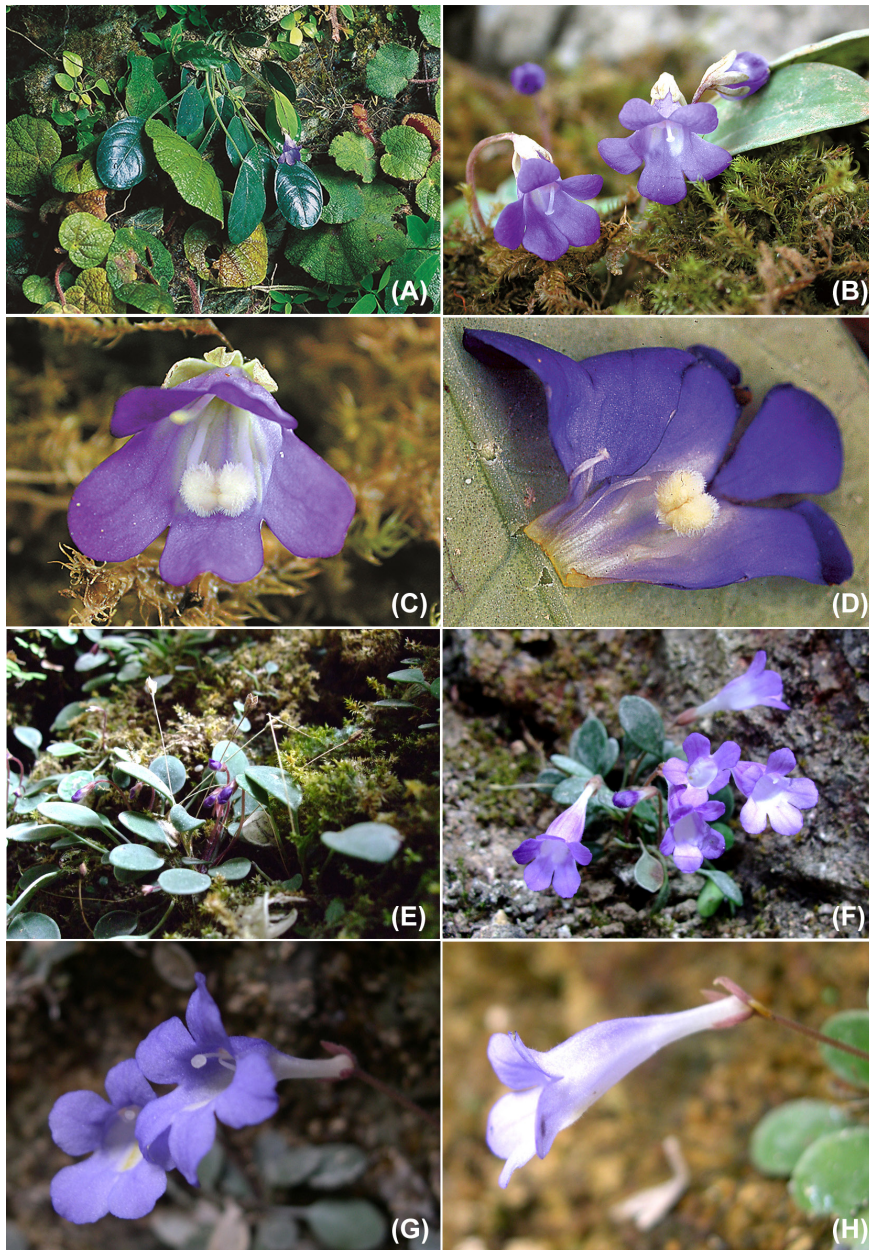


Figure 4. Living plants of *Litostigma crystalinum* and *L. coriaceifolium*. (A–B) Habit, (C) corolla in front view, (D) corolla open stamens and staminodes, (E–F) habit, (G) corolla in front view, (H) corolla in lateral view. (A–D) *Litostigma crystalinum* (photos taken by Yu-Ming Shui based on type material of *L. crystalinum*, (E–H), *L. coriaceifolium* (photos taken by Fang Wen based on type material of *L. coriaceifolium*).

convex beneath, inconspicuous; petiole (3–)5–15 cm long, glabrous. Cymes 1–3, 1-flowered; peduncle 5–7 cm long, glabrous; bracts 2, small, linear, ca 3 mm long, ca 0.5 mm wide. Sepals 5, lanceolate, ca 5 mm long, ca 1 mm wide, slightly pubescent outside, glabrous inside, acute at apex. Corolla purplish blue, infundibuliform, 2.2–2.4 cm long, slightly pubescent outside, glabrous inside, with two yellow lines in the throat; corolla tube 1.0–1.2 cm long with short and thick proximal undilated part; adaxial lobes 2, rounded, 6 mm long, ca 9 mm wide, abaxial lobes 3, obovate, ca 10 mm long, ca 10 mm wide. Stamens 2 in anterior position,

adnate to 3 mm above the base of the corolla tube; filaments linear, slightly erect, ca 10 mm long; anthers elliptic, ca 2 mm long, sparsely short pilose, coherent adaxially. Staminodes 2, ca 3.5 mm long, glabrous, adnate to 3–4 mm above the corolla base. Disc annular. Ovary ellipsoid, ca 1.5 mm long, pubescent, 1-loculed with 2 parietal and intrusive placentae; style ca 10 mm long, glabrous. Stigma bilobed, branches ca 1 mm long. Fruit 4–5 mm long, narrowly ovoid, glabrous, dehiscent into 4 valves. Seeds ovoid, obliquely reticulate with raised testa cell walls, ca 0.3 mm long, ca 0.1 mm wide.

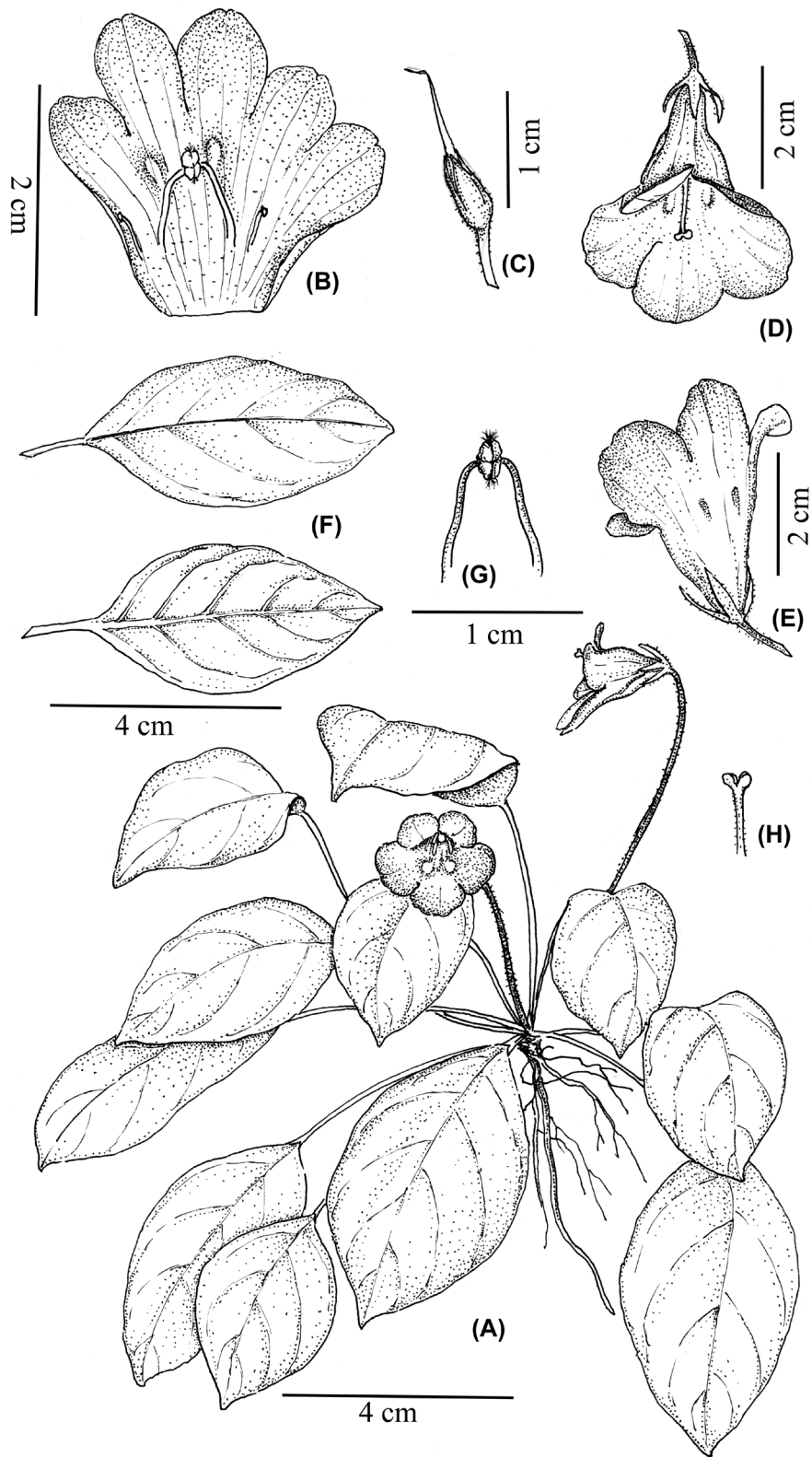


Figure 5. *Litostigma napoense* sp. nov. (A) Habit, (B) corolla open with stamens and staminodes, (C) calyx and pistil, (D) corolla in front view, (E) corolla in lateral view, (F) leaf blade, (G) stamens, (H) stigma. (Drawn by Bing-Mou Wang based on Y. F. Huang & Y. Nong 79975).

Phenology

Flowering from November to December; fruiting time unknown.

Distribution and habitat

Litostigma napoense is presently known only from two populations, one in Napo county, Guangxi Guangxi Zhuangzu

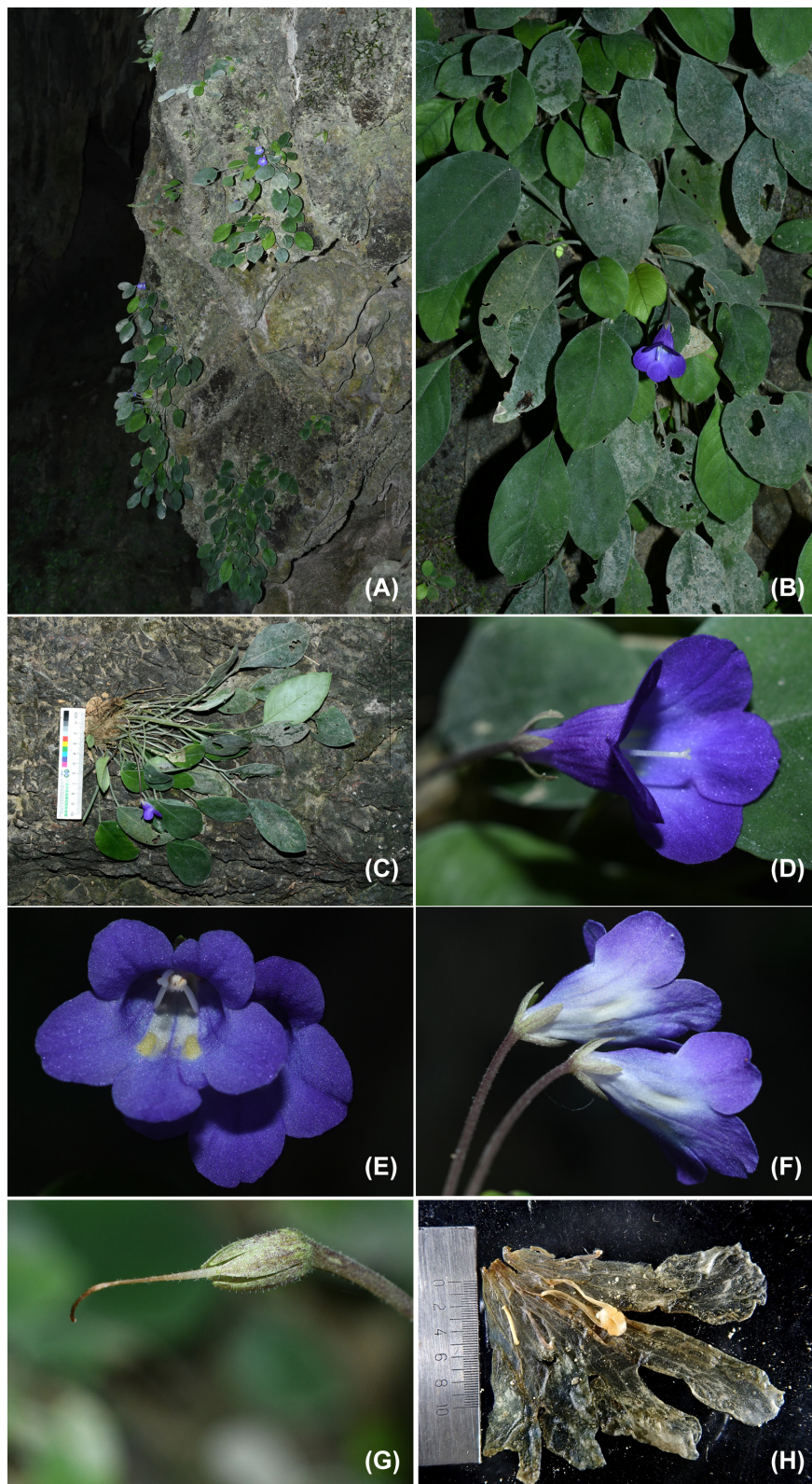


Figure 6. Living plants of *Litostigma napoense* sp. nov. (A) Habitat, (B–C) habit, (D) corolla in upper view, (E) corolla in front view, (F) corolla in lateral view, (G) calyx and pistil, (H) corolla open showing stamens and staminodes. (Photos taken by Yun-Feng Huang based on Y. F. Huang & Y. Nong 79975).

Autonomous Region of China and another population in northern Vietnam. It grows on the cliff at the entrance of limestone caves at an elevation of 500–850 m a.s.l.

Conservation status

Litostigma napoense is presently known only from two populations with a small number of individuals. The population in Napo is relatively small, with only about 200 individuals. This species may easily be threatened by habitat loss. Due to the small size of the populations, this new species should be considered Critically Endangered (CR)[B1ab(iii, v)] based on the IUCN red list criterion (IUCN 2019).

Similar species and phylogenetic position

Litostigma napoense is most similar to *L. crystallinum* Y. M. Shui & W. H. Chen (Wei et al. 2010, p. 181) (Fig. 4A–D) in habit, shape and texture of leaves, shape and color of corolla, lanceolate sepal, anthers elliptic, pilose and coherent adaxially, but differs by having larger leaf blades (3.0–9.0 cm long, 1.7–4.3 cm wide versus 4.5–6.0 cm long, 3.0–4.0 cm wide), leaf apex acute to obtuse (versus rounded), lateral nerves inconspicuous (versus distinct), corolla 2.2–2.4 cm long, with two yellow lines in the throat (versus 1.5–1.7 mm long, with two white lines in the throat), and stigma bilobed (versus disciform form). The phenology of the two species

is also quite different: the flowering period of *L. napoense* is from November to December, but that of *L. crystallinum* is in May. Lu et al. (2020) reported *L. crystallinum* flowering in December from northern Vietnam, which is different from the type from Malipo county, Yunnan Province of China. The identity of *L. crystallinum* from Vietnam needs further study.

A key to species of *Litostigma* (distribution map is shown in Fig. 7)

- 1a. Leaf blade thick papery; undilated part of corolla tube short and thick; anthers sparsely short pilose.
 - 2a. Leaf blade apex acute to obtuse; lateral nerves inconspicuous; corolla 2.2–2.4 cm long; stigma bilobed.....*L. napoense*
 - 2b. Leaf blade apex rounded to obtuse; lateral nerves distinct; corolla 1.5–2.1 mm long; stigma disciform*L. crystallinum*
- 1b. Leaf blade coriaceous; undilated part of corolla tube long and slender; anthers prominently villous.
 - 3a. Leaf blade 1.0–2.1 cm long, 0.6–1.1 cm wide; petiole 0.4–2.5 cm long, sparsely pubescent; peduncle 1.5–2.0 cm long, pubescent; corolla with two yellow lines in the

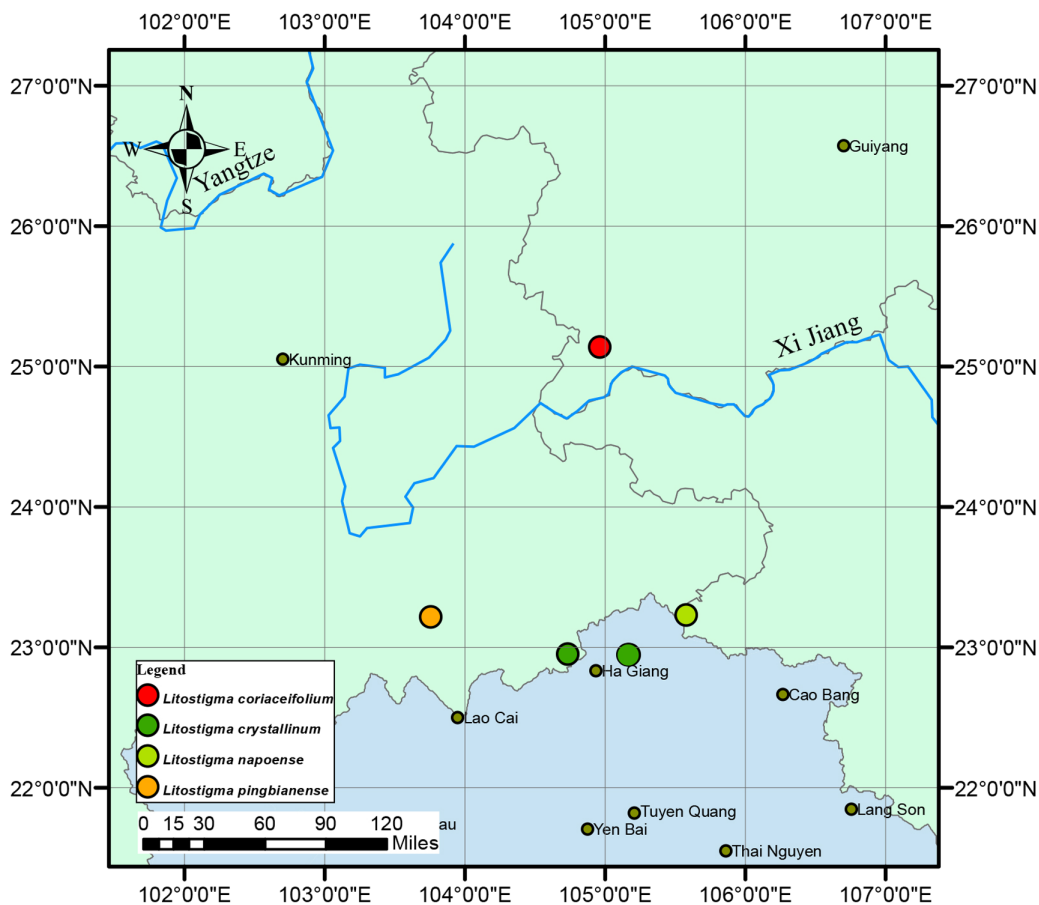


Figure 7. Distribution of the genus *Litostigma*.

throat, pubescent outside; style ca 9.5 mm long; filaments erect, 3.0–4.0 mm long; fruit 5.0–6.0 mm long, glabrous.....*L. coriaceifolium*
 3b. Leaf blade 1.5–4.0 cm long, 0.7–1.9 cm wide; petiole 1.5–9.0 cm long, glabrous; peduncles 2.5–5.0 cm long, glabrous; corolla with two white lines in the throat, glabrous outside; style ca 12 mm long; filaments arcuate upward to the adaxial lip, 5.0–6.0 mm long; fruit 3.0–4.0 mm long, pubescent..... *L. pingbianense*

Data availability statement

Data available from the Dryad Digital Repository: <<http://dx.doi.org/10.5061/dryad.sn02v6x3d>> (Chen et al. 2020b).

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Author contributions

You-Sheng Chen: CRediT contribution not specified. **Yun-feng Huang:** Investigation (equal). **Lian-sheng Xu:** CRediT contribution not specified. **Bing-Mou Wang:** CRediT contribution not specified.

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