



***Primulina sinovietnamica* (Gesneriaceae), a new species identified by both morphological and molecular characters from the limestone area in Guangxi, China**

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Abstract

A new species of Gesneriaceae from Southwest Guangxi, China is described here as *Primulina sinovietnamica*. This previously unknown species is morphologically similar to *P. pungentisepala* and *P. ningmingensis*, but can be distinguished from the latter two taxa by several non-overlapping morphological characters. This hypothesis is consistent with the molecular phylogenetic tree based on sequences of the chloroplast *matK* gene, confirming evolutionary affinity of the new species to the two morphologically similar taxa. The three sampled individuals from the two presently known populations of the new species cluster together in the phylogenetic analysis, separated from the other two closest taxa by two common base substitutions, confirming the recognition of this new species.

Introduction

Primulina Hance was described as a monospecific genus in the diverse family Gesneriaceae, which long remained to comprise only the single species *Primulina tabacum* Hance (1883: 169). It was presumed to be related to *Chirita* Buch.-Ham. but *P. tabacum* differs in having unusual salverform flowers with an almost actinomorphic patent limb (Weber *et al.* 2011). The generic status of *Primulina* was challenged by later studies in which *P. tabacum* was placed in *Chirita*, as they have similar morphological traits in stigma and rosette habit with opposite leaves, and the unusual salverform flower was assumed to be possibly resulting from a pollinator shift from bee to butterfly (Wang *et al.* 1990, 1998, Li & Wang 2004). Recent molecular studies further suggested that *Primulina tabacum* was deeply embedded in the section *Gibbosaccus* of *Chirita*, while the genus *Chirita* (as well as its major sections including *C.* section *Gibbosaccus*) was polyphyletic, which at least represented five lineages of independent origin (Wang *et al.* 2011, Weber *et al.* 2011). In the reorganising of *Chirita* and its allied genera based on molecular phylogenies, *Chiritopsis* W.T.Wang, *Wentsaiboea* D.Fang & H.D.Qin (except *W. tiandengensis* Yan Liu & B.R.Pan (2010: 739), a species found to be more closely related to *Lagarosolen* W.T.Wang [Möller *et al.*, 2011]) and the large number of species described in section *Gibbosaccus* of genus *Chirita* were transferred to *Primulina* due to its taxonomic priority, and this monotypic genus henceforth was expanded dramatically to accommodate over 130 taxa (Weber *et al.* 2011). The genus *Chirita* was subsequently abandoned.

The expanded *Primulina*, as well as other lineages of Gesneriaceae, are still growing because of the numerous new species that are being constantly discovered. Within the past several years, at least dozens of new species of *Chirita*, particularly in the section *Gibbosaccus* (of which most taxa have been recently transferred into *Primulina*) have been described mostly from the main centre of diversity in South China (e.g.

Xu *et al.* 2008, Li & Möller 2009, Huang *et al.* 2010, Li & Zhu 2010, Xu *et al.* 2010, Huang *et al.* 2011, Wu *et al.* 2011, Xu *et al.* 2011a, 2011b), and it is expanding rapidly with morphologically distinct new species more recently described (e.g. Hong *et al.* 2012, Huang *et al.* 2012, Wu *et al.* 2012, Xu *et al.* 2012). Although the Gesneriaceae has great morphological diversity, the molecular evidence has shown large discrepancies with the traditional taxonomy and suggested that the taxonomic units, probably at generic as well as specific level, could have been overestimated by the synonyms and too trivial division of the species or populations with slightly morphological variations into different genera or species (Weber *et al.* 2011, Christenhusz 2012). Therefore, it is necessary to combine molecular evidence with morphological taxonomy and study both morphological as well as genetic diversity.

In the present study, we describe another new species of Gesneriaceae, which can be morphologically distinguished from all other known species in the family. This new species was initially found by the first author at a site in the limestone areas of Guangxi Zhuang Autonomous Region near the border between China and Vietnam during a floristic survey in August 2009, and was then collected when in flower in October 2009. Subsequently, another morphologically similar population was discovered in Longgang National Nature Reserve in the same limestone region. After widely reviewing the related literature (e.g. Li & Wang 2004, Wei *et al.* 2010) and comparison with available herbarium specimens, these two populations were assumed to represent a new species in the genus *Primulina*. To avoid the possibility that the taxonomy and diversity recognition of the new species were misled by potentially confusing morphological convergence as exemplified by previous cases, e.g. *Wentsaiboea tiandengensis* and *W. luochengensis* Yan Liu & W.B. Xu (2010: 739) were found to be not related to each other, but closer respectively to *Lagarosolen* and *Primulina* as shown by molecular studies (e.g. Möller *et al.* 2011), we generated sequences of the DNA barcode chloroplast *matK* gene from three sampled individuals of the two new populations, as well as from some other close taxa. These were amplified and used in the present study to identify its closest relatives and to test the hypothesis of these two populations forming a single species belonging to the genus *Primulina*. The detailed characters of this new species and its evolutionary relationships to other closely related taxa are described and illustrated below.

Material and methods

We sampled three individuals from the presently known two populations of the new species (respectively from Longgang National Nature Reserve and Chunxiu Nature Reserve, over 30 kilometers between the two places), two accessions of *Primulina ningmingensis* and three accessions of *P. pungentisepala* (both from Longgang National Nature Reserve), and some other *Primulina* taxa, which are morphologically similar to the new species, or from a geographically similar area, because previous phylogenies implied a high correlation between evolutionary affinity and geographic distribution. Finally, a total of 54 accessions of 48 species were collected, all from Guangxi, south China, where they are endemic or chiefly distributed. They include 41 species in *Primulina* and seven outgroup species from *Beccarinda* Kuntze, *Henckelia* Spreng., *Lagarosolen*, *Paraboea* (C.B. Clarke) Ridl. and *Paraisometrum* W.T. Wang.

Total genomic DNA was extracted from silica-gel dried leaves using the CTAB protocol (Doyle & Doyle 1987). DNA sequences of the chloroplast *matK* genes were amplified based on a PCR procedure of initial denaturation at 94°C for 3 min, followed by 34 cycles of 30 sec at 94°C, 40 sec at 52°C, and 90 sec at 72°C, with extension at 72°C for 10 min. PCR products were purified using TIANgel Midi Purification Kit (Tiangen Biotech) according to the manufacturer's instructions and then sequenced on an ABI 3730 DNA Sequencer (Applied Biosystems International, Foster City, CA, USA), using Big Dye Terminator (Applied Biosystems, Shanghai, China). The primers used for amplification and sequencing included: *matK*-AF (CTATATCCACTTATCTTTCAGGAGT) and *matK*-8R (AAAGTTCTAGCACAAGAAAGTCG) designed for Gesneriaceae.

DNA sequences were manually edited and spliced using the software Bioedit according to the bidirectional chromatograms for each of the sequences. All sequences after edition were aligned in Clustal X 1.81 (Thompson *et al.* 1997) and adjusted manually in Bioedit 5.0.9 (Hall 1999). The flank regions at both ends with many missing sites were excluded and the indels within sequences were filled by gaps. The final matrix is available upon request from the corresponding author. Phylogenetic analyses were conducted based on Maximum Parsimony (MP) in PAUP version 4.0b10 (Swofford 2002) and Bayesian Inference (BI) in MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001). For the MP analysis, 100 replicates of random stepwise addition with tree bisection-reconnection (TBR) branch swapping were performed using heuristic searches, with all most-parsimonious trees saved at each replicate (MulTree on). Support for each branch was assessed using bootstrap analysis with 100 bootstrap replicates, each with 10 stepwise additions. As the inadequacy of the parsimony informative sites of the *matK* sequences would have yielded excessive equally most-parsimonious trees in the tentative MP heuristic search, therefore, 500,000 trees were set as the maximum limit for the first round of heuristic searches based on the original matrix and 50,000 maximum trees were set for each of the bootstrap pseudoreplications. Prior the Bayesian analysis, we used Modeltest (Posada & Crandall 1998, Posada & Buckley 2004) to select the fittest DNA substitution model based on the Akaike Information Criterion (AIC). Two runs of Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses were performed simultaneously, with a random starting tree and four chains for each run (one cold and three heated). The MCMCMC length was 10,000,000 generations and the chain was sampled every 1000th generation from the cold chain. Bayesian clade posterior probabilities and average branch lengths were calculated based on the sampled trees combined from the two runs after the first 15% of the total trees were discarded as burn-in. The burn-in for each run was validated by plotting the likelihood values against the generations.

Results of the molecular analysis

The *matK* region varied from 950 bps to 1156 bps for all the sampled 54 accessions with an average GC content of 32% and the final aligned matrix had 1156 bps in length with 95 informative sites as well as 132 additional variable but uninformative sites. TVM+G model was selected as the fittest substitution model for the *matK* gene by the Modeltest.

Both MP and Bayes analyses yielded concordant majority-rule consensus trees with the Bayesian consensus tree better resolved (Fig. 3). In the MP analysis, the maximum constraint of 500,000 MP trees with 304 steps length (CI = 0.83 and RI = 0.90) was hit at the first random addition replication. The present MP and Bayesian consensus trees from the chloroplast *matK* gene are overall consistent with those obtained from the chloroplast *trnL-F* and nuclear ITS sequences as in the previous molecular studies. The sampled different accessions of the three morphologically close species respectively cluster into its own species with moderate or high support (BS = 85% and PP = 0.99 for the new species; BS = 100% and PP = 1.00 for *P. pungentisepala*; BS = 100% and PP = 1.00 for *P. ningmingensis*). The three species constitute a monophyletic lineage in contrast to the rest, within which the new species forms the sister to *P. pungentisepala* (BS = 84%; PP = 1.00), followed by *P. ningmingensis* (BS = 100%; PP = 1.00). Further examination of the DNA sequences also found two common base substitutions for the three accessions of the new species which are different from *P. pungentisepala* and *P. ningmingensis*, and one additional base substitution that is endemic to the accession of the new species from Longgang (Table 1).

TABLE 1. Synapomorphic and autapomorphic base substitutions of the three accessions of the new species to the closest relatives.

	346	501	887
<i>Primulina sinovietnamica</i> (Longgang)	?	C	C
<i>Primulina sinovietnamica</i> (Chunxiu1)	C	A	C
<i>Primulina sinovietnamica</i> (Chunxiu2)	C	A	C
<i>Primulina pungentisepala</i> (Longgang 1)	T	A	T
<i>Primulina pungentisepala</i> (Longgang 2)	T	A	T
<i>Primulina pungentisepala</i> (Longgang 3)	T	A	T
<i>Primulina ningmingensis</i> (Longgang 1)	T	A	T
<i>Primulina ningmingensis</i> (Longgang2)	T	A	T

The first row represents the site numbers in the matrix.

The mark (?) represents ambiguous state for the site.

Base substitutions of the three samples of the new species are highlighted in bold.

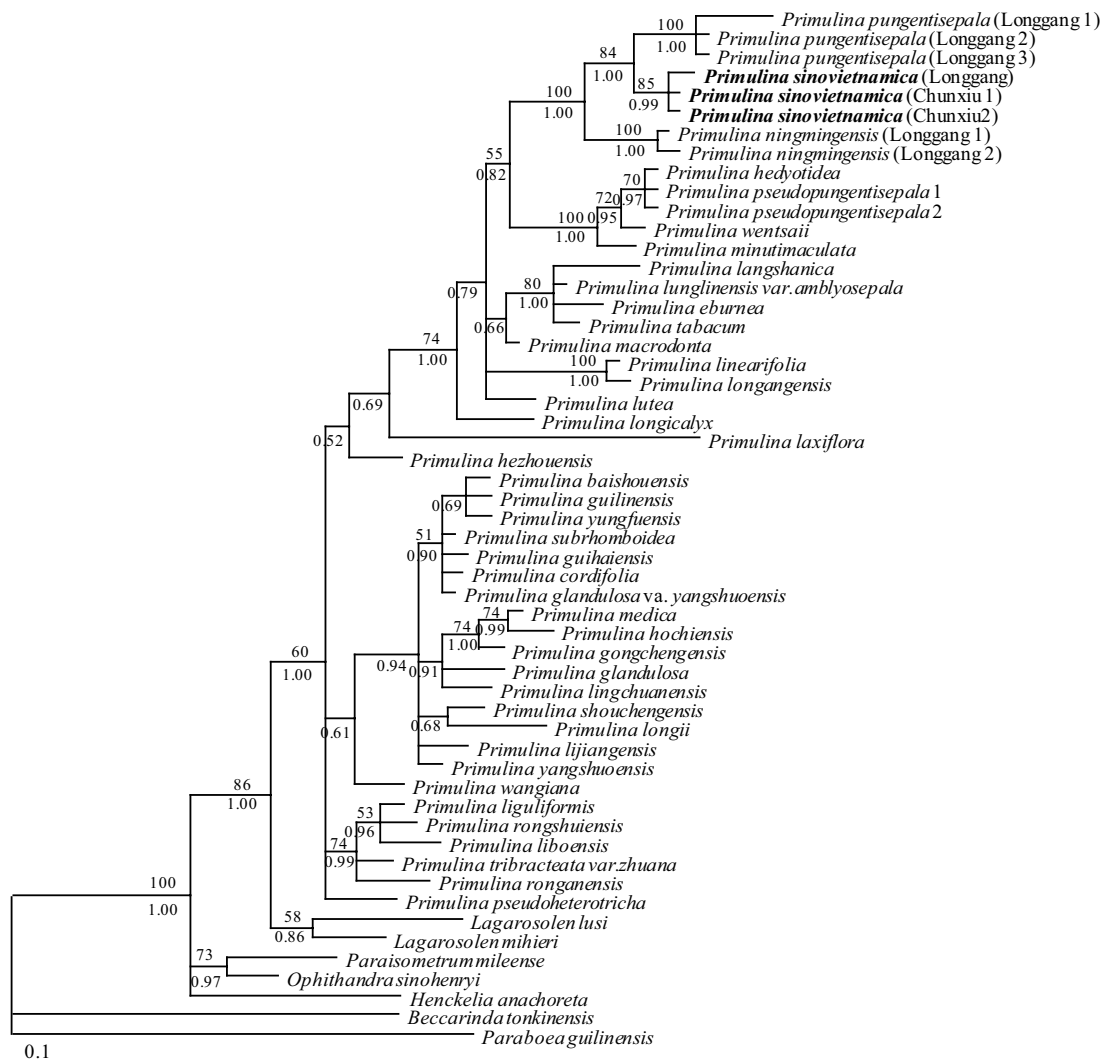


FIGURE 1. Bayesian majority-rule consensus phylogram based on the sequences of the *matK* gene, with both posterior probabilities and MP bootstrap supports bigger than 50% mapped to the branches. The three accessions representing the new species are highlighted in bold.

Taxonomic treatment

Primulina sinovietnamica W.H.Wu & Q.Zhang, *sp. nov.* (Fig. 1 & Fig. 2 A–D, H–K)

A new species similar to *P. pungentisepala* and *P. ningmingensis*, but it differs from these species in having purple villous leaf surfaces, minute bracts (<2.5 cm vs. >5 cm), corolla small (1.2–1.8 vs. 3.0–3.5), pale purple without stripes (vs. corolla white or red with two yellow stripes) and purple (vs. white) stamen filaments.

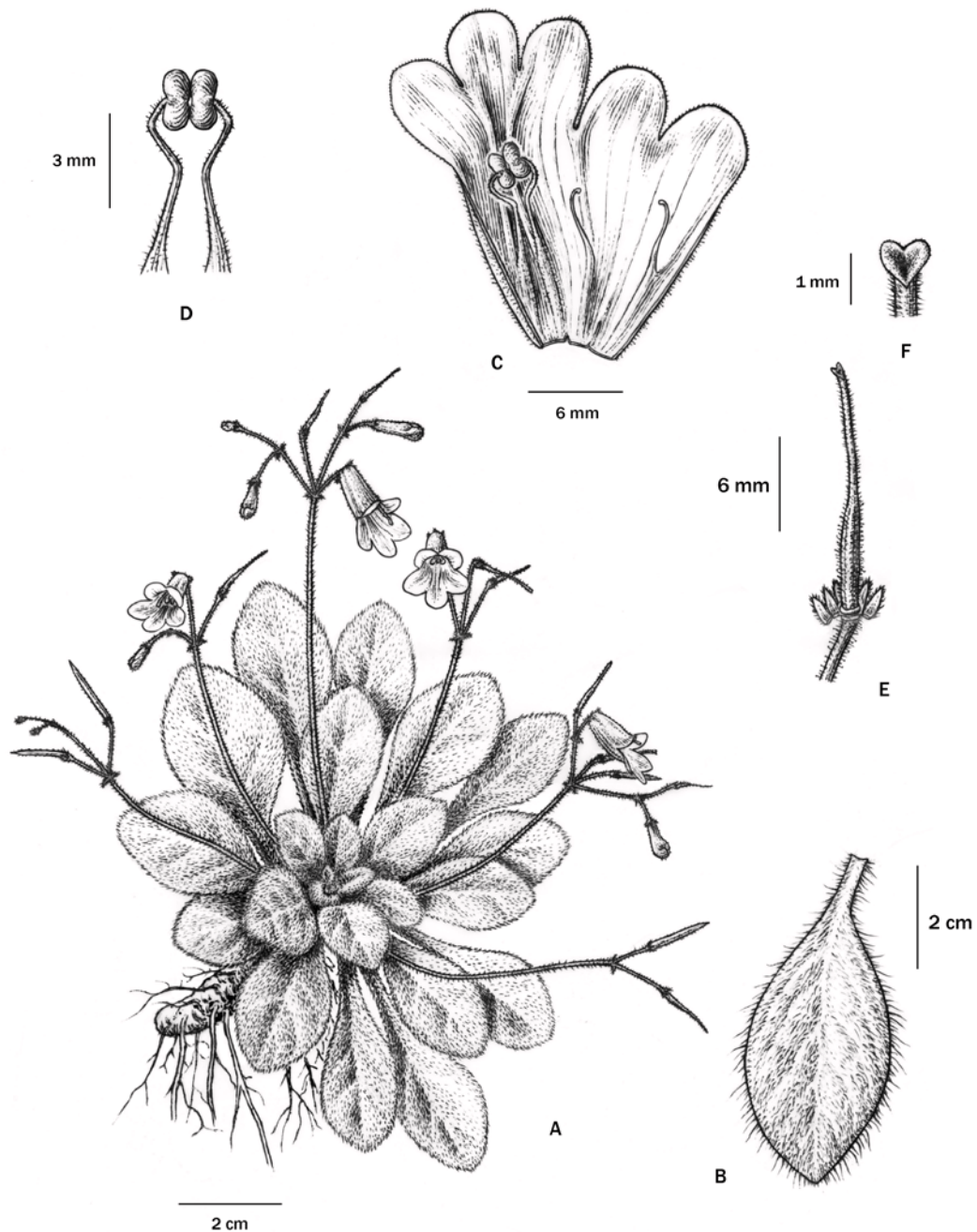


FIGURE 2. *Primulina sinovietnamica*. A) habit; B) leaf; C) opened corolla; D) stamens; E) pistil and opened calyx; F) stigma. Drawn by Yun-Xi Zhu.

Type:—CHINA. Guangxi: Longzhou County, Longgang National Nature Reserve. 595 m, 22°30'54.03" N, 106°52'14.29" E, 30 October 2010, *Wang-Hui Wu W0321* (holotype IBK!, isotype IBK!).

Perennial herbs. Rhizomes subterete, 2–9 × 0.5–1.6 cm. Leaves petiolate; petioles appanate, 1–4 cm long, 1–3 mm wide, appressed purple villous; leaf blade thick-papery when dry, 1.8–5 × 0.8–3 cm, elliptic, ovate to oblanceolate, appressed purple villous on both surfaces, the bases cuneate to decurrent, the margins entire, the apices acute to obtuse, lateral veins 2–4 on each side of midrib. Inflorescences cymose, 5–12 cymes per plant, axillary, 1–2-branched, 7–22-flowered; peduncle 4–10 cm long, 1.5–2.1 mm wide, scattered purple villous; bracts 2, 1.5–2.5 × 0.3–0.9 mm, lanceolate, appressed purple villous, the margins entire, the apices acute; pedicel 0.8–2.5 cm long, ca. 1 mm wide, spreading villous. Calyx 5-parted nearly to the base, 0.8–1.2 × ca. 0.5 mm, lobes linear-lanceolate, appressed villous outside, glabrous inside, the margins entire, the apices acuminate. Corolla pale purple, 1.2–1.8 cm long, pubescent outside, sparsely pubescent inside, the tube 0.8–1.3 cm long, 4–6 mm in diameter at the mouth, ca. 2.4 mm in diameter at the base; limb distinctly 2-lipped, adaxial lip 2-lobed to over middle, the lobes 2–3 × ca. 3.5 mm, oblong, the abaxial lip 3-lobed to near middle, the lobes 3–4 × ca. 4 mm, oblong. Stamens 2, adnate to ca. 5 mm above the corolla base; filaments ca. 6 mm long, purple, sparsely pubescent, strongly geniculate at 3.5 mm above the filament base, anthers ca. 2 mm long, glabrous, barbate abaxially. Staminodes 3, lateral ones ca. 4 mm long, apices capitate, purple, glabrous, adnate to 5 mm above the corolla tube base, middle one 0.3–0.6 mm long, adnate to 5 mm above the corolla tube base. Disc ca. 0.9 mm in height, margin orbicular, glabrous. Pistil 1.0–1.4 cm long, ovary 4–7 mm long, ca. 1 mm wide, mixed pubescent and glandular-pubescent; style 5–8 mm long, ca. 0.6 mm wide, mixed pubescent and glandular-pubescent; stigma cordiform, apex retuse, 0.5 mm wide, 2-lobed. Fruit a straight capsule, 1.3–2 cm long.

The other ecotype of the new species above is also shown in Fig. 2 D, I, K, which differs from the first one (B–C, H, J in Fig. 2) by a leaf pubescence with long white or purple hairs.

Ecology, phenology and etymology:—Only two populations have been found for the new species, one at the peak of a limestone hill in Longgang National Nature Reserve and the other on the precipice of a limestone hill in Chunxiu Nature Reserve, both from Longzhou County, Guangxi, China. It flowers in September to November. The specific epithet is derived from the type locality, around the border of China and Vietnam.

Distinguishing characters:—*Primulina sinovietnamica* is morphologically close to *P. pungentisepala* (Fig. 2 E, L) and *P. ningmingensis* (Fig. 2 F, M), but it can be distinguished from the latter two by a few qualitative and quantitative characters in leaf, bract, calyx, staminodes and stigma, as shown by the detailed morphological comparisons of the three species in Table 2.

TABLE 2. Morphological comparisons of *Primulina sinovietnamica* with *P. pungentisepala* and *P. ningmingensis*

	<i>Primulina sinovietnamica</i>	<i>Primulina pungentisepala</i>	<i>Primulina ningmingensis</i>
leaf	purple villous	upper surface pilose, lower surface densely puberulous	pubescent
cymes	5–12, 7–22-flowered	2–4-flowered	5–10, 8–20-flowered
bracts	1.5–2.5 × 0.3–0.9 mm, villous	5–13 × ca. 1.5 mm, pubescent	8–25 × 4–11 mm, pubescent
pedicel	0.8–2.5 cm long, villous	2.7–5 cm long, glandular puberulous	1–3 cm long, glandular-pubescent
calyx	outside villous	outside pubescent	outside glandular-pubescent
corolla	pale purple, 1.2–1.8 cm long, stripe absent	white, ca. 3.5 cm long, two yellow stripes	light red, 3–3.5 cm long, two yellow stripes
filaments	purple sparsely pubescent	white glabrous	white glandular-pubescent
staminodes	3, lateral ones glabrous	2, glabrous	3, lateral ones sparsely pubescent
stigma	cordiform	suboblong	cordiform
flowering time	September to November	April to May	August to September

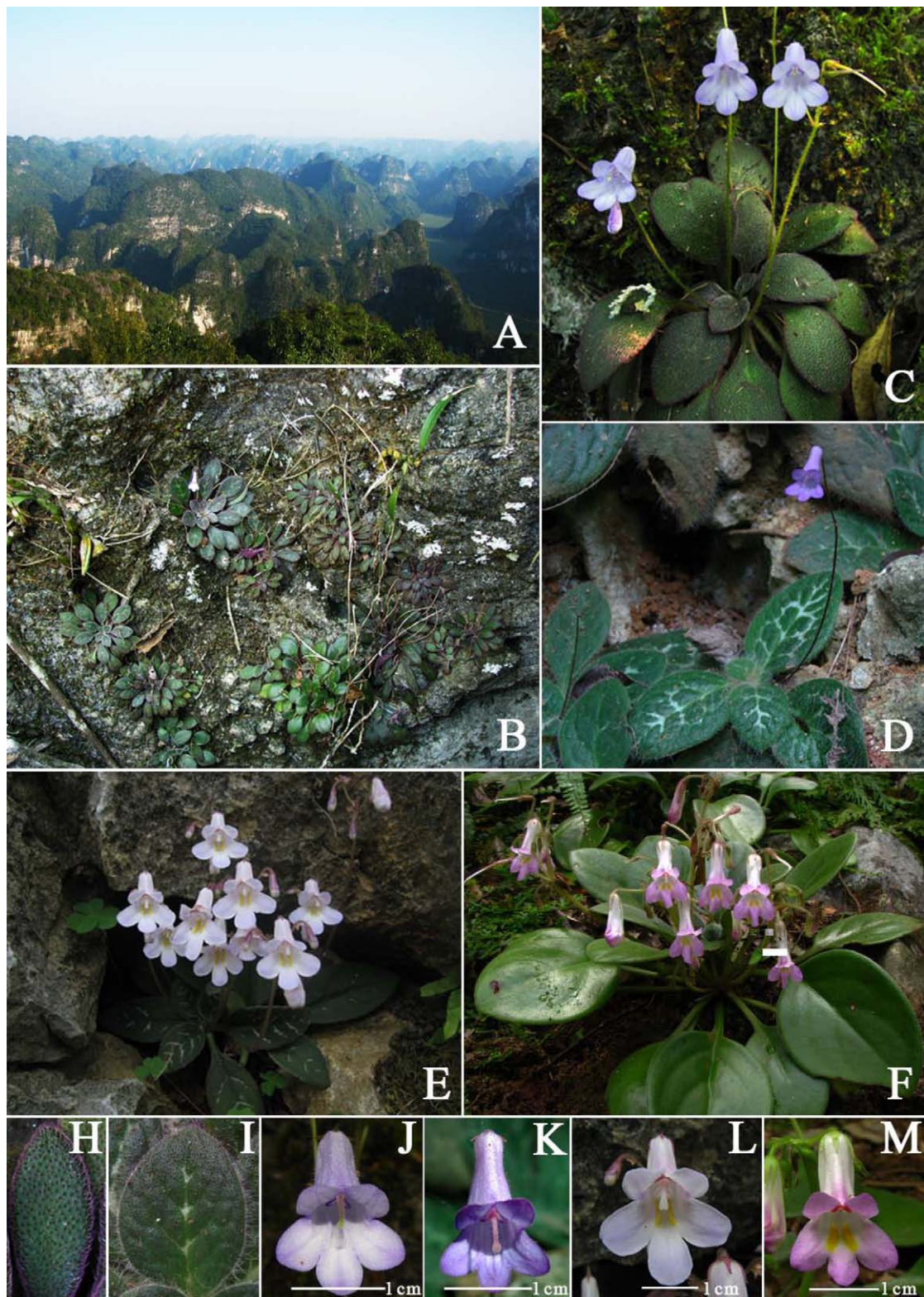


FIGURE 3. A–C, H, J) The ecotype of *Primulina sinovietnamica* in Longgang National Nature Reserve. A) overview of the landscape; B) habitat; C) habit; H) leaf surface; J) flower. D, I, K) The ecotype of *P. sinovietnamica* in Chunxiu Nature Reserve. D) habit; I) leaf surface; K) flower. E, L) *P. pungentisepala*. E) habit; L) flower. F, M) *P. ningmingensis*. F) habit; M) flower.

Additional specimens examined (paratype):—CHINA. Guangxi: Longzhou County, Chunxiu Nature Reserve, on the precipice of a limestone hill, 22°25'22.02" N, 106°38'30.08" E, 288 m, 19 October 2010, *Yu-Song Huang Y0027* (IBK!).

Discussion

In this study, we identified and described a new Gesneriaceae species based on both morphological and molecular evidence. Several non-overlapped qualitative and quantitative morphological characters, the different accessions respectively clustering together in three groups for the three close taxa, and two common base substitutions of the *matK* gene of the new populations compared with its presently known closest relatives suggest that two newly found populations most likely represent a new species. The genealogical separation of the geographically close populations among *Primulina pungentisepala*, *P. ningmingensis* and *P. sinovietnamica* in Longgang National Nature Reserve, in addition to the striking differences of the potential pollination-related floral characteristics, e.g. different corolla colour and the absent honey guides (yellow stripes) inside the corolla for the new species, would also imply the populations of the different species are probably reproductively isolated. Although the two newly found populations have also shown some morphological variations and one additional base substitution (the individual from Longgang population as shown in table 1) between them, we conservatively treat them as a single species in the present study pending additional and unambiguous evidence to indicate they are reproductively isolated.

In addition to the other taxa recently described in *Primulina*, this new species has expanded the known diversity of this diverse lineage from its Asian distributional centre of southern China and northern Vietnam. In regard to the complex topography of the limestone regions and narrow ranges of highly specialised habitats (e.g. caves, crevices, precipices) on limestone substrate for most of the Gesneriaceae plants, more undescribed species are still expected to be discovered in future field investigations in these environs.

On the other hand, recent molecular studies have largely disapproved of the traditional generic classifications in the family, which were assumed to be not only resulted from high morphological homoplasy or heterogeneously morphological evolution, but also from too trivial classification of the morphological variations (Möller *et al.* 2011, Weber *et al.* 2011). The species were also speculated to be overestimated due to excessive division of the different populations with minor morphological change into different species. Therefore, the molecular evidence should be combined with morphological data to recognize the diversity and evaluate the traditional taxonomy, as well as trace the evolutionary relationships within Gesneriaceae in future studies.

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