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Primulina nymphaeoides (Gesneriaceae), a new species from Guangxi, China

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ABSTRACT: *Primulina nymphaeoides*, a new cave-dwelling species of Gesneriaceae from the limestone region of southwestern Guangxi, China is described and illustrated. Morphologically, it is most similar to *P. longzhouensis*, but differs by having some easily distinguished characteristics, for example different leaf blade indumentum, obviously fewer flowers on one cyme, bigger flower size, and different stigma shape, and so on. Furthermore, the phylogenetic analysis of two DNA region ITS and *trnL-F* sequence including 179 taxa of *Primulina* s.l. has been provided. Both Bayesian inference (BI) and maximum likelihood (ML) analyses show the similar supported tree topology, indicating that *P. nymphaeoides* is recovering as one of species in a phylogenetic polytomy closed to *P. longzhouensis* and other species. A Global Species Conservation Assessment classifies *P. nymphaeoides* as Critically Endangered (CR).

KEY WORDS: Cave-dwelling, flora of Guangxi, ITS, *Primulina*, *Primulina longzhouensis*, limestone flora, taxonomy, *trnL-trnF*.

INTRODUCTION

All species of the genus, *Primulina* Hance (Gesneriaceae), are perennial herbaceous plants, and this genus now exhibits the most diversity in the Chinese Gesneriaceae, including approximately 123 species and 8 varieties of *Primulina* s.l. after the revision (Wang *et al.*, 2011; Weber *et al.*, 2011). As of November 2022, there were 227 species (excluding infraspecific taxa) (GRC, 2022) in the genus. China is the center of diversity for *Primulina* with at least 204 species and 15 varieties occurring there at present (Wen *et al.*, 2022), especially in limestone areas (e. g. Wei, 2018; Xin *et al*., 2018; Wen *et al.*, 2019; Ge *et al.*, 2020; Liu *et al.*, 2020; Xin *et al.*, 2020a, b, c, 2021; Xu *et al.*, 2021; Zhang *et al.*, 2021). The tropical and subtropical karst limestone mountainous areas in Guangxi are the centers of species diversity and differentiation of this genus (Li *et al.*, 2019).

With the help of a local guide, staffs of Gesneriad Conservation Center of China (GCCC) and Guangxi Institute of Botany, CAS, discovered an unknown species of *Primulina* in Longzhou County, Guangxi during field investigations in 2019. At that time, these flowers of this unknown *Primulina* species were long past their prime, and its vegetative morphology felt a bit like the published *P. longzhouensis* (B.Pan & W.H.Wu) W.B.Xu & K.F.Chung. Therefore, some living plants were introduced to the National Gesneriaceae Germplasm Resources Bank of GXIB (NGGRB) and GCCC nursery in 19 Oct., 2019, and they were planted together with *P. longzhouensis* from then on. In the homogenous garden, the two were cultivated in the same cultivation condition. This unknown plant bloomed twice from May to August 2020 and 2021. Because we found it looks different to *P. longzhouensis*, we re-visited the type locality for observe living plants and collect specimens in May, 2020. We found that it is obviously different from *P. longzhouensis*, and the two can be easily distinguished (Fig. S1). Therefore, two individuals were made into specimens for reference. After consulting related literature, monographs (Wei *et al*., 2010), local plants checklist of Guangxi (Wei, 2018) and Chinese Flora (Wang *et al*., 1990, 1998), we believe that it can be well distinguished from the known *Primulina* spp. (including *P. longzhouensis*, particularly), so we describe it as a new species to science, namely *P. nymphaeoides* Y.G.Wei & W.C.Chou.

MATERIAL AND METHODS

All available specimens of the new species were deposited at the herbarium of Guangxi Institute of Botany (IBK) and the herbarium of National Taiwan University (TAI). All morphological characters were studied based on the material from field and herbarium specimens using a dissecting microscope (Olympus CX23, Tokyo, Japan). The morphological characters of the type specimens were compared with the protologue descriptions of all previously described *Primulina* species in monographs and local floras (Wang, 1998; Li and Wang, 2007; Wei *et al.*, 2010; Wei, 2018).

Genomic DNA was extracted from fresh or dried materials using a modified CTAB protocol (Chen *et al.*, 2014). The plastid *trnL-F* intron spacer (*trnL-F*) and the nuclear ribosomal internal transcribed spacer (ITS) region

Table 1. Statistics for the molecular datasets used in this study.

	ITS	*trnL-trnF*	Combined all
No. of sequences (ingroup/outgroup)	179/2	179/2	179/2
Aligned length (bp)	756	830	1586
Length variation (bp)	613–643	716–777	1345–1386
Variabe characters (bp)	470	203	673
Parsimony-informative characters (bp)	366	91	457
Model selected (ML)	TPM3+I+G4	K3Pu+F+G4	K3Pu+F+I+I+R3
Model selected (BI)	K2P+I+G4	GTR+F+G4	HKY+F+I+G4

were used as molecular markers as previously done by Weber *et al.* (2011). DNA extraction, PCR amplification and sequencing of the new species followed Möller *et al.* (2009, 2011). To elucidate the phylogenetic affinities of the new species, ingroup (179 species of *Primulina*) and outgroup (two of *Petrocodon*) were chosen based on recent phylogenetic analyses (Kang *et al.*, 2014; Xu *et al.*, 2019; Ding *et al.*, 2021; Gong *et al.*, 2022), and from which sequences were available from GenBank (Table S1). Visual comparison of optimal tree topologies generated by ML analysis of *trnL-F* and ITS datasets to compare topological inconsistencies. Conflicts between tree topologies was considered significant when the inconsistent topologies all receive ≥80% of the bootstrap values (Fu *et al.*, 2022).

We performed phylogenetic analyses of *Primulina* based on combined data-set of *trnL-F* and ITS sequences using Bayesian inference (BI) and maximum likelihood (ML). For BI analysis, we employed MrBayes v.3.2.6 (Ronquist *et al.*, 2012) to obtain a maximum clade credibility (MCC) tree. The matrix of ITS and *trnL-F* sequence was aligned by MAFFT. Bayesian inference was performed using one million generations, four runs, four chains, a temperature of 0.001, 25% trees discarded as burn-in, and trees sampled every 1,000 generations (1,000 trees sampled in total) with HKY+F+I+G4 model. We conducted the ML analysis using IQ-TREE ver. 2.0.6 (Nguyen *et al.*, 2015) with 1,000 bootstrap replicates (Hoang *et al.*, 2018), and defaulted ModelFinder (Kalyaanamoorthy *et al.*, 2017) and found K3Pu+F+I+I+R3 as the best-fit substitution model. Tree visualization was achieved in FigTree v1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Characteristics and statistics of the datasets used in this study are summarised in Table 1. Visual inspection showed no significant topological contradictions for bootstrap support consistency between *trnL-F* and ITS datasets (suppl. Figs. S2, S3), thus these regions were combined in our analysis.

The aligned matrix of *trnL-F* and ITS sequence was 1586 characters. Of the 673 (42%) variable characters, 457 (28.8%) were parsimony-informative, including indels. BI and ML analyses showed the undescribed species as belonging to a strongly supported clade (BP = 95%, PP = 0.82) that included *P. crassirhizoma* (Zhao *et al.*, 2013), *P. longzhouensis* (Pan *et al.*, 2010), *P. ningmingensis* (Yan Liu & W.H.Wu) W.B.Xu & K.F.Chung (Wu *et al.*, 2011), *P. pungentisepala* (W.T.Wang) Mich.Möller & A.Weber (Li *et al.*, 1997), *P. diffusa* Xin Hong, F.Wen & S.B.Zhou (Zhou *et al.*, 2014), *P. jingxiensis* (Yan Liu, W.B.Xu & H.S.Gao) W.B.Xu & K.F.Chung (Xu and Liu, 2009) and *P. petrocosmeoides* B.Pan & F.Wen (Pan and Wen, 2014) (Fig. 1).

TAXONOMIC TREATMENT

Primulina nymphaeoides Y.G.Wei & W.C.Chou, ***sp.nov.*** **靜蓮報春苣苔 Fig. 2**

Type*:** China. Guangxi Zhuangzu Autonomous Region, Chongzuo City, Longzhou County, Xinlian Village, growing on moist limestone rock surfaces of a Karst cave. Alt. ca. 324 m, 22°30'N, 106°53'E, 29 May 2020, *Bi Nong-Sheng & Chou Wei-Chuen, BNS & CWC190529-01* (Holotype***: IBK! IBK00445393; ***Isotypes***: IBK! IBK00445394; TAI).

***Diagnosis*:** *Primulina nymphaeoides* is morphologically similar to *P. longzhouensis*, but can be easily distinguished from the latter by leaf blade adaxial surface densely puberulent and abaxial sparely puberulent (*vs.* appressed pilose on both surfaces), lateral veins 2–3 on each side of the midrib, but they are inconspicuous on the leaves of wild plants. (*vs.* inconspicuous), bracts lanceolate to narrowly oblong (*vs.* lanceolate), calyx lobes size 6–7 × 1.8–3 mm (*vs.* 3–4 × ca. 0.8 mm) and outside glandular-puberulent, inside glabrous (*vs.* outside glandular-puberulent, inside sparsely puberulent), corolla 22–27 mm long (*vs.* 10–14 mm long), corolla tube ca. 13 mm long (*vs.* 8–9 mm long) and adaxial lip ca. 6 × 4 mm, lobes slightly oblique ovate (*vs.* 2.5–3 × 2–2.5 mm, lobes oblong or rounded), filaments ca. 8 long (*vs.* ca. 4 mm long), pistil ca. 16 mm long (vs. 7–9 mm long) and ovary ovoid (*vs.* narrowly ovoid) (Fig. S1).

Perennial herbs. Rhizomatous stem subterete, ca. 5 mm long, 2–3 mm in diam. Leaves 12–18, all basal forming a rosette, or crowded at the apex of rhizome after long period of growth; petiole 2–7 cm long, 2–4.5 mm in diam., densely white pubescent; leaves herbaceus, blade green, occasionally yellow-green, fleshly, blades elliptic or broadly elliptic to suborbicular, 2–5 × 1–3 cm, with adaxial surfaces densely puberulent and abaxial nearly glabrous, base attenuate to cuneate, margin entire, apex obtuse to subacute; lateral veins 2–3 on each side of the midrib, prominent abaxially, inapparently impressed adaxially. Cymes 3–8, axillary, 1–2-branched, 2–10-flowered; peduncle 3–8 cm long, densely spreading white pubescent; Bracts 2, opposite, lanceolate to narrowly oblong, 5–10 × 1–2 mm, margin entire, abaxially densely

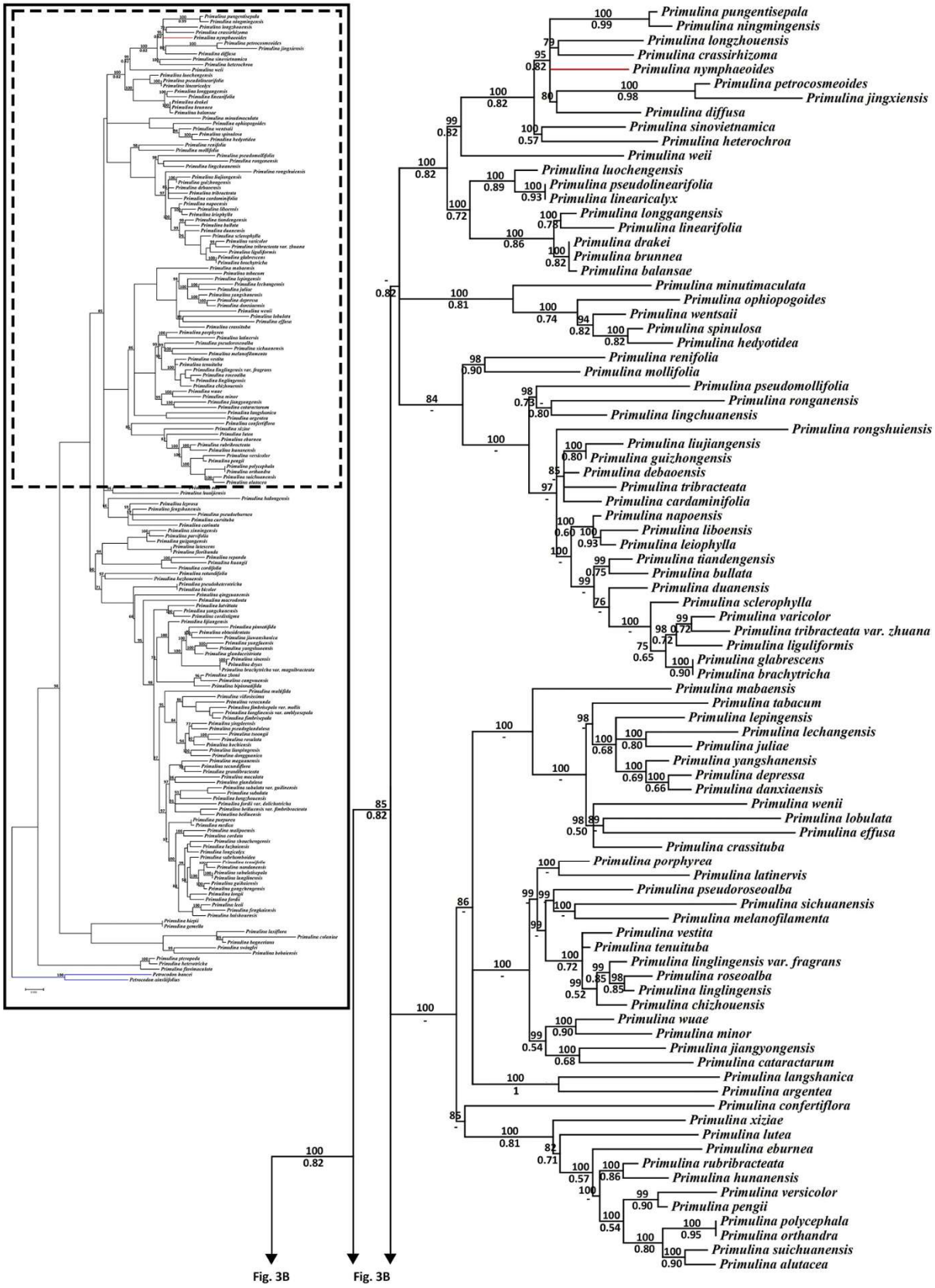

Fig. 1. Continued

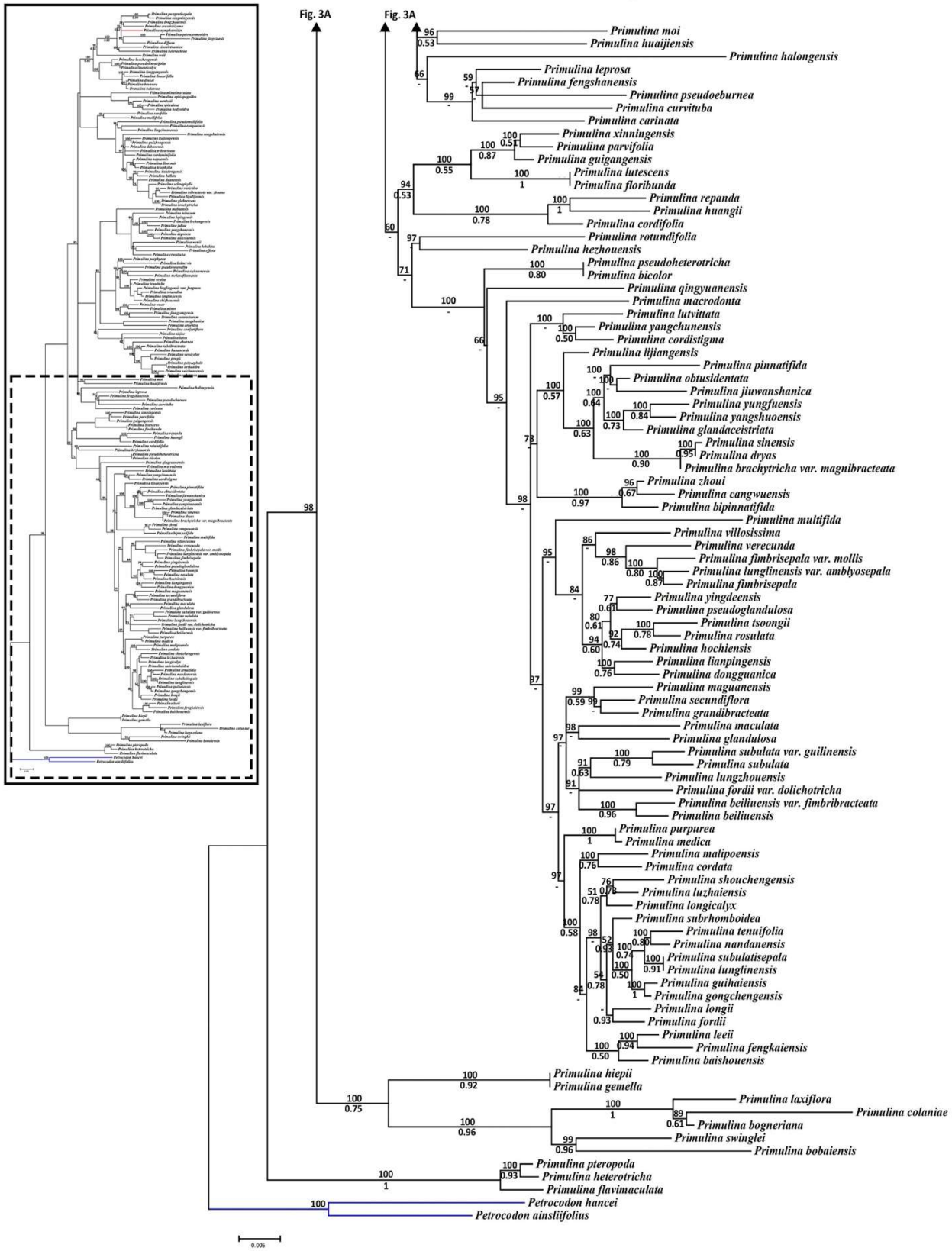

Fig. 1. Phylogenetic tree of ***Primulina*** generated by Bayesian inference (BI) of the combined dataset (ITS, *trnL-F* spacer). Numbers on branches indicate posterior probability (≥0.5) of BI and bootstrap values (≥50%) of maximum likelihood analyses.

Table 1. Morphological comparison of ***Primulina nymphaeoides*** and ***P. longzhouensis***.

Characters	***P. nymphaeoides***	***P. longzhouensis***
Leaf blade	adaxial surfaces densely puberulent and abaxial nearly glabrous	appressed pilose on both surfaces
Lateral veins	2–3 on each side of the midrib	inconspicuous
Calyx	6–7 × ca. 1 mm, outside puberulent, inside glabrous	3–4 × ca. 0.8 mm, outside glandular-puberulent, inside sparsely puberulent
Corolla	22–27 mm long, corolla tube ca. 13 mm long, adaxial lip ca. 6 × 4 mm, lobes slightly oblique ovate	10–14 mm long, corolla tube 8–9 mm long, adaxial lip 2.5–3 × 2–2.5 mm, lobes oblong or rounded
Filament	ca. 8 mm long	ca. 4 mm long
Anther	ca. 2 mm long	ca. 1.3 mm long
Staminode	lateral ones ca. 4 mm long, adnate to ca. 5.5 mm above the corolla tube base	lateral ones 1–3 mm long, adnate to ca. 2.5 mm above the corolla tube base
Pistil	ca. 16 mm long, ovary ovoid, ca. 5 mm long, ca. 1.5 mm in diam.	7–9 mm long, ovary narrowly ovoid, ca. 3.5 mm long, ca. 0.8 mm in diam.
Style	ca. 11 mm long	4–5 mm long
Stigma	obtrapeziform	triangular

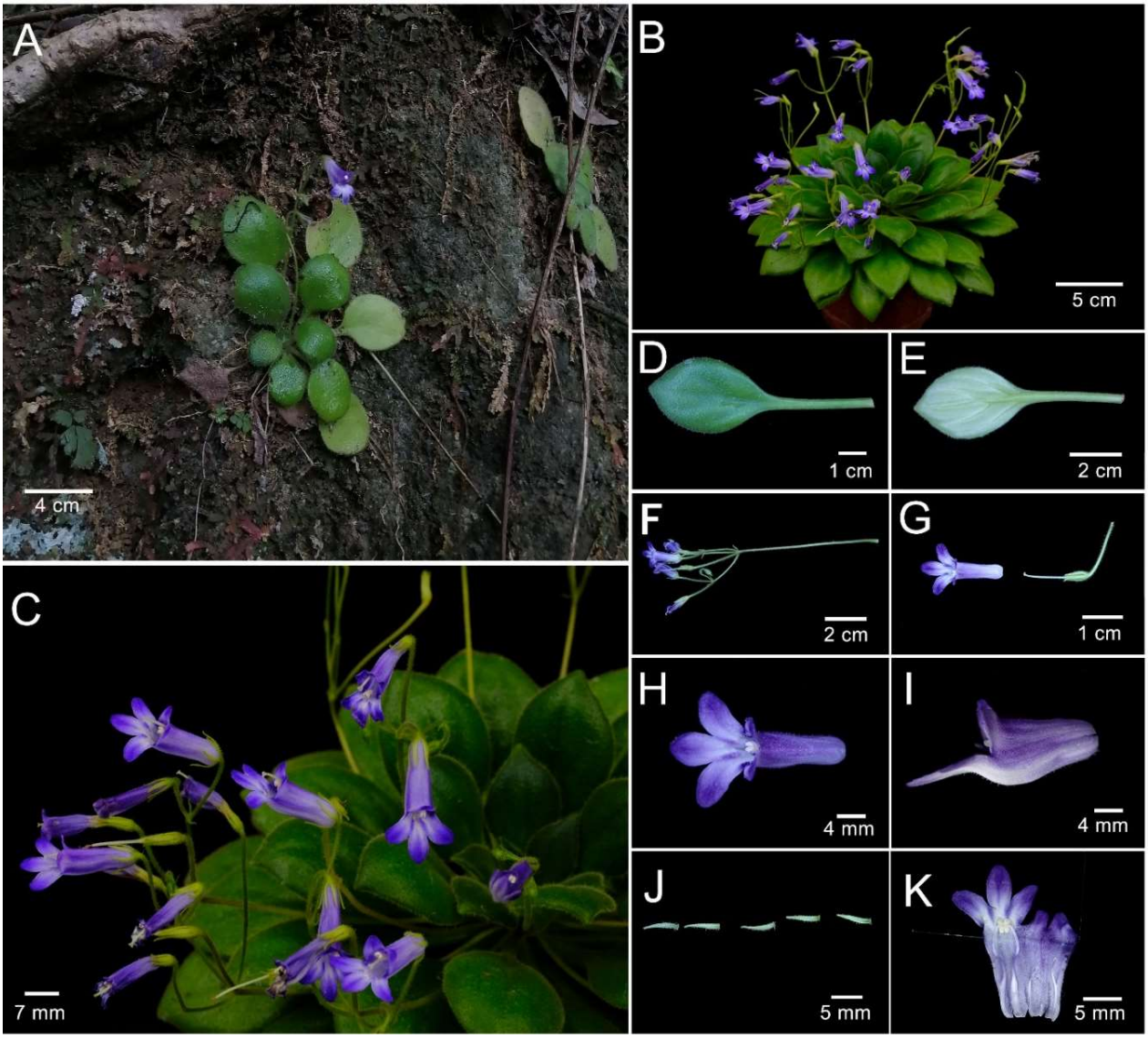

Fig. 2. ***Primulina nymphaeoides*** sp.nov. **A**: Habitat. **B**: Habit. **C**: Flower. **D**: The adaxial surface of leaf blades. **E**: The abaxial surface of leaf blades. **F**: Cyme. **G**: Corolla, pistil and calyx. **H**: Top view of corolla. **I**: Side view of corolla. **J**: Calyx. **K**: Opened corolla for showing stamens and staminodes.

pilose and glandular puberulent, adaxially nearly glabrous. Pedicel 1–3 cm long, ca. 1 mm in diam., densely eglandular and glandular puberulent. Calyx 5-parted from base, lobes lanceolate-linear, 6–7 × ca. 1 mm, apex acute, outside puberulent, inside glabrous, margin entire. Corolla brightly purple, ca. 2.5 cm long, outside pubescent and glandular puberulent, inside glabrous. Corolla tube funnelform, ca. 1.3 cm long, ca. 4.8 mm in diam., constricted at the mouth; limb 2-lipped, adaxial lip 2-lobed to near base, lobes slightly oblique ovate, ca. 6 × 4 mm, apex rounded; abaxial lip 3-lobed to the middle or more, lobes ovate-oblong, ca. 3 × 2 mm, apex rounded. Stamens 2, adnate to ca. 4 mm above the corolla tube base; filaments linear, pale purple, ca. 8 mm long, geniculate from above the middle, sparsely glandular puberulent, anthers reniform, yellowish-white, ca. 2 mm long, ca. 1.5 mm in diam., glabrous, adhering to each other. Staminodes 3, lateral ones ca. 4 mm long, apex capitate, pale purple, glabrous, adnate to ca. 5.5 mm above the corolla tube base, middle one ca. 0.4 mm long, white, glabrous. Disc ca. 0.9 mm height, glabrous, margin entire. Pistil ca. 1.6 cm long, ovary ovoid, densely pubescent and glandular puberulent, ca. 5 mm long, ca. 1.5 mm in diam., style ca. 11 mm long, ca. 0.6 mm in diam., linear, sparsely puberulent and glandular puberulent. Stigma obtrapeziform, ca. 0.8 mm long. Capsule oblong-oval, ca. 7 mm long, ca. 2 mm in diam., densely pubescent and glandular puberulent, style commonly persistent when capsule mature, dehiscing predominantly on one side.

***Phenology*:** Flowering from May to August, fruiting from August to October.

***Etymology*:** The specific epithet is derived from the numerous rosette leaves of this species look like green water lily flower, especially after years of cultivation.

***Distribution and Ecology*:** *Primulina nymphaeoides* is only found in Longzhou County, Chongzuo City, Guangxi, China. It only grows in the crevices or moist rock surface at the entrance of limestone cave (Fig. S4) in the subtropical evergreen seasonal rain forest at an altitude of ca. 324 m.

***Conservation status*:** In 2019, we observed approximately 200 mature individuals of this new species in the type locality, but there were no more than 50 mature individuals in 2021. The severe reduction in population size is mainly caused by human disturbance. Because of its gorgeous flower color and dense leaves, this plant has been over-collected by the locals and sold as an ornamental plant. The main threat now comes from environmental damage caused by grazing, and there is a risk of poaching in the future because its distribution is close to the villages. Thus, following the IUCN Red List Categories and Criteria (IUCN, 2022), it is temporarily assessed as Critically Endangered [CR B1 ab (iii, v) +B2ab(iii,v)]. However, our field surveys of this species are insufficient and more field work is needed to fully understand its geographic distribution.

***Note*:** Our analyses of DNA sequence data suggest that *Primulina nymphaeoides* recovers in a polytomy including *P. crassirhizoma*, *P. diffusa*, *P. jingxiensis*, *P. petrocosmeoides*, *P. longzhouensis*, *P. ningmingensis*, and *P. pungentisepala* (BP = 95%). Of these, the new species most closely resembles *P. longzhouensis* from which it can be distinguished based on leaf blade, lateral eins and cyme etc. as summarized in Table 1.

Additional specimen examined*: *Primulina longzhouensis (B.Pan & W.H.Wu) W.B.Xu & K.F.Chung: CHINA. Guangxi, cultivated in Guilin Botanical Garden, introduced from Shuikou Town, Longzhou County, in the crevice of limestone hills, 4 Dec. 2009, *Bo Pan & Wang-Hui Wu 091751*; Guangxi, Longzhou County, Xiadong Township, close to Longqin Tunnel, growing on the limestone hill, 22 Jul. 2011, *Xu Wei-Bin 11229*.

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